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Yeast Chromosome Replication and Segregation

CAROL S. NEWLON

Department of Microbiology and Molecular Genetics, UMDNJ-New Jersey Medical School, Newark, New Jersey 07103

INTRODUCTION

Eucaryotic chromosomes, each of which consists of a single linear deoxyribonucleic acid (DNA) molecule and its associated chromosomal proteins, are replicated and transmitted to daughter cells with high fidelity during mitotic cell cycles. For example, in the yeast *Saccharomyces cerevisiae*, the rate of mitotic chromosome loss is approximately 10^{-5} per cell division (105, 142, 256). *S. cerevisiae* has a number of important advantages for the study of eucaryotic DNA replication and chromosome segregation. In contrast to "larger" eucaryotes, its chromosomal DNAs are small, with molecules ranging in size from approximately 250 upward to 2,000 kilobases (kb) (60–62, 77, 223, 297, 330). The average yeast chromosomal DNA is only four times the mass of bacteriophage T4 DNA and about 100-fold smaller than a *Drosophila* or mammalian chromosome. The small size of yeast chromosomes allows molecular studies of intact chro-

mosomal DNAs. Yeast has long been a favorite genetic system, and the ability to isolate and characterize mutants defective in the complicated processes of DNA replication and chromosome segregation is a powerful tool. In addition, recombinant DNA technology and the characteristics of yeast DNA transformation make it possible to move or delete specific segments of chromosomes with ease and to study any chromosomal segment of interest in relative isolation on autonomously replicating plasmids.

This review focuses on our current understanding of yeast chromosomal DNA replication and mitotic segregation. I begin with replication of intact chromosomes and then consider the DNA sequences and proteins that are likely to be required for replication. I then discuss the recent work that has defined the structure of two other *cis*-acting elements required for normal chromosome function, centromeres and telomeres. Finally, I consider the requirements for the efficient mitotic segregation of chromosomes. Other recent reviews cover certain aspects of the topics covered here (31, 58, 81, 108, 112, 118, 197, 268, 276, 390, 399).

A Word about Chromosome Structure

Although a detailed consideration of chromosome structure is beyond the scope of this review, it is important to remember that about half the mass of a chromosome is protein. The very long chromosomal DNAs are condensed through several orders of packaging to fit inside the nucleus. The first order of packaging is the association of DNA with the four core histones H2A, H2B, H3, and H4 to form the nucleosomal structure of the 10-nm chromatin fiber. The next level of organization, the 30-nm fiber, is dependent on histone H1 (reviewed in reference 168). There is evidence that, for both metaphase chromosomes and interphase chromosomes, the 30-nm chromatin fiber is organized in radial loop domains that include 30- to 150-kb lengths of DNA anchored at their ends to metaphase chromosome scaffolds or, in the case of interphase nuclei, to the nuclear lamina or the nuclear matrix. The attachment sites for these radial loop domains are probably specific, and each loop is topologically constrained. Evidence from both nuclease digestion experiments and autoradiography is consistent with the site of DNA replication being at or near the matrix (100, 281). While its role(s) is presently not well defined, DNA packaging has the potential for affecting DNA replication and chromosome segregation. In fact, when the core histone pairs are synthesized in unbalanced ratios, chromosome loss rates increase (256).

Studies of yeast chromosome structure are not as extensive as for higher eucaryotes. The 10-nm chromatin fiber of *S. cerevisiae* is organized into nucleosomal subunits typical of other eucaryotes (reviewed in reference 133). *S. cerevisiae* apparently does not have an H1 histone, so it is unlikely that it contains a 30-nm chromatin fiber (67). Nuclear matrices similar to those of higher eucaryotes have been prepared from *S. cerevisiae* (33, 82, 84, 307, 308). Newly synthesized DNA is more closely associated with these matrices than bulk DNA, consistent with the notion that DNA replication may occur at or near the matrix (307). The question of whether specific DNA sequences are preferentially associated with the matrix is not completely resolved. Cockerill and Garrard have reported that a matrix association sequence from the mouse kappa light-chain immunoglobulin gene binds specifically to isolated yeast nuclear matrices (82). In addition, yeast *ARS* and *CEN* elements have recently been reported to bind specifically to nuclear scaffold preparations (3). However, another group found no evidence for the attachment of specific DNA sequences to these structures (307). Moreover, when plasmid DNA binding to matrices was examined, binding was correlated with plasmid size and not with DNA sequences (84). Thus, depending on preparation conditions, it is possible to see or not to see specific binding. Further studies are needed to clarify this issue.

CHROMOSOMAL DNA REPLICATION

Spacing of Replication Origins

DNA replication usually proceeds bidirectionally from sites known as origins of replication. A replicon is defined as the DNA replicated from a single origin. Most procaryotic chromosomes consist of a single replicon. In all eucaryotes studied, chromosomal DNAs contain multiple replicons (reviewed in reference 136). Replicating yeast molecules visualized by electron microscopy often contain multiple internal replication bubbles or terminal replication forks or both (277, 280, 298, 299). In addition, DNA fiber autoradiography experiments have revealed that replication proceeds bidirectionally from replication origins (300, 320).

Although replicons are certainly heterogeneous in size, estimates of average replicon size can be obtained from measurements of center-to-center distances between replication bubbles. The average spacing found in data obtained from both electron microscopy and DNA fiber autoradiography is approximately 90 kb. However, this estimate is biased for three reasons. Initiation of replication is not synchronous. Therefore, early in the replication of a chromosomal DNA molecule, the spacing between replication bubbles is an overestimate of the spacing between origins because not all origins have initiated. Later in S phase, adjacent replication bubbles fuse, also leading to an overestimate of the size of a replicon. Finally, replication structures that are far apart can be more easily separated by DNA breakage than those close together. This leads to a disproportionate loss of long replicons. An analysis of replicon spacing as a function of extent of replication can be used to correct for the first two factors (37, 413). When this correction was applied to a sample of small replicating chromosomal DNAs in which breakage was not a major factor, an origin spacing of 36 kb was calculated (277). This estimate of replicon size is substantiated by the distribution of center-to-center distances in raw data from electron microscopic measurements and fiber autoradiography. Although the average replicon spacing is 90 kb, there are several peaks of center-to-center distances. The shortest center-to-center spacings define a peak that lies between 30 and 45 kb in most studies (277, 280, 298–300). A similar estimate of replicon size has been obtained by measuring the size of nascent daughter DNA strands, using alkaline sucrose gradient sedimentation (186). Using 36 kb as an estimate of average origin spacing, there are approximately 400 replication origins in *S. cerevisiae* chromosomal DNA.

The important questions of whether chromosomal replication origins are at specific DNA sequences and, if so, how many classes of origins exist are discussed in the section on autonomously replicating sequences.

Timing in Cell Cycle

As in other eucaryotes, chromosomal DNA replication in *S. cerevisiae* occurs during S phase of the cell cycle. The

duration of S phase and its timing relative to other events in the cell cycle have been studied by using whole-cell autoradiography (14, 45, 319, 398) and flow microfluorometry (179, 344). S phase occupies 25 to 50% of the cell cycle in strains grown with glucose as a carbon source and with excess nitrogen. When the length of the cell cycle is increased by growth in a poor carbon source, the G1 phase is expanded (14, 344). In contrast, when growth rate is decreased by nitrogen limitation, an equivalent expansion of either all phases of the cell cycle (319) or of G1 and S phase (179) is observed. In daughter cells, which have longer cell cycles than mother cells, it is the G1 phase that is expanded (45). Thus, both the absolute length of S phase and the fraction of the cell cycle it occupies can be manipulated experimentally.

It is useful to be able to relate the timing of S phase to some easily scored landmark in the cell cycle such as bud emergence and growth. In the strain used for the first such study, the beginning of S phase coincided with bud emergence (398), and it has been widely assumed that this correlation is true for all strains. However, additional studies have demonstrated that bud emergence occurs before or at the beginning of S phase in some strains (14, 45, 398) and as late as halfway through S phase in others (45, 319). Therefore, care must be taken to establish this correlation for the particular strain being used.

Temporal Structure of S Phase

Because replication of procaryotic chromosomes is usually initiated from a single origin, the time at which a particular DNA sequence replicates depends upon its distance from the origin (e.g., see reference 26). In contrast, multiple replication origins may allow both the distance from an origin and the relative time or efficiency of initiation at the origin to influence the time at which a DNA sequence replicates. From measurements of the sizes of adjacent replication structures in DNA fiber autoradiographs and electron micrographs, most adjacent origins in yeast cells appear to be activated at about the same time (277, 320). However, up to 20 to 30% of adjacent origins are activated more than 10 min apart and some are activated more than 20 min apart during an S phase of approximately 40 min (320). Therefore, initiation at origins must occur for at least the first half of S phase.

The first approach used to examine whether specific DNA sequences replicate at specific times during S phase in *S. cerevisiae* was to determine the susceptibility of particular genes to mutation by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. In *Escherichia coli*, this mutagen is believed to act preferentially at the replication fork (154). In *S. cerevisiae*, S-phase cells were found to be more sensitive to nitrosoguanidine than G1-phase cells, consistent with the selective mutagenesis of replicating DNA (56, 88, 338). Measurements of reversion frequencies of mutations in several genes as a function of the time of mutagenesis during S phase suggested that different genes replicate at different times during S phase. Each gene showed maximal reversion at a particular time, and these times were significantly different for three of five genes analyzed in one study (56) and for five of six genes analyzed in a second study (338).

Direct measurements of the time of replication of specific sequences have been made by using density transfer experiments on synchronized cultures (107, 248). Newly replicated DNA of hybrid density was separated from unreplicated fully dense DNA by centrifugation in CsCl gradients. The kinetics of appearance of particular DNA sequences in newly replicated DNA were examined by hybridizing fractions from each gradient with radiolabeled cloned sequences. In the first study (107), the replication of the chromosomal copies of *ARS1* (from chromosome IV) and 10Z (from chromosome V) were shown to occur at distinguishably different times, with *ARS1* replicating early and 10Z replicating late. A third sequence, containing *ARS2*, may have replicated at an intermediate time. In addition, a plasmid containing *ARS1* replicated during the same interval as the chromosomal *ARS1* sequence. This observation suggests that either the timing information is carried in the cloned *ARS1* fragment or the "default" replication time is early in S phase.

Additional density transfer experiments defining the time of replication of 34 chromosomal sequences demonstrated that each replicated during a reproducible interval of S phase (248, R. M. McCarroll, Ph.D. thesis, University of Washington, Seattle, 1988). In a sample of eleven randomly chosen DNA sequences, four replicated early, five replicated in mid-S, and two replicated late. The time interval from the beginning of replication of the earliest fragment to the end of replication of the latest fragment was about the same as the length of S phase determined from incorporation of radioactive precursors. These experiments demonstrate directly that there is a temporal program of replication in *S. cerevisiae*.

In addition to random sequences, the time of replication of 9 of the 16 centromeres and of 5 telomere-adjacent unique sequences was examined (248). All centromeres replicated early and five of the six telomere-adjacent sequences replicated late, suggesting that each chromosome arm contains at least one early replicating and one late replicating region.

The pattern of replication of the cloned 200-kb region of chromosome III is consistent with predictions based on the analysis of fragments from other regions of the genome. Most of the region, which extends from the left telomere to the *MAT* locus at about the midpoint of the right arm (279), replicates during the first half of S phase. A transition from early to late replication occurs approximately 30 kb from the left telomere. The *HMR* locus, near the right telomere, also replicates late (McCarroll, Ph.D. thesis; R. M. McCarroll, A. Reynolds, W. L. Fangman, and C. S. Newlon, manuscript in preparation). Thus, chromosome III appears to contain a central early replicating domain separated from late replicating domains at each end. Within the early replicating region, adjacent origins must begin replication at similar times, consistent with the earlier finding based on analysis of random DNA molecules (277, 320).

Two important questions remain unresolved. First, what controls the timing of replication? It could be regulated at the level of initiation, with some origins initiating early and others late. The timing of initiation could then be regulated by interaction of proteins with *cis*-acting "timing" sequences or by the structure or location of a chromosomal segment within the nucleus. Alternatively, late replicating sequences could replicate late simply because they are far away from an active origin and a long time is required for a fork to reach them. The transition from early to late replication on the left arm of chromosome III is gradual and is consistent with a single fork moving from an origin that initiates early to the telomere. However, late initiating origins cannot be eliminated by the data.

Second, are there distinct boundaries between early and late replicating regions? A priori, if an origin that initiates early were separated from an origin that initiates late by the average interorigin distance, then forks from the "early"

origin would have to be prevented from replicating the "late" origin by something that slows or blocks replication fork movement. It has recently been shown that there is a strong block to replication fork movement near the 3' ends of the actively transcribed ribosomal ribonucleic acid (RNA) genes in yeast cells (47, 228). In addition, the *cis*-acting terminus of replication found in the *E. coli* chromosome is in a region where replication forks encounter the 3' ends of actively transcribed genes (44, 91, 153). Thus, one potential barrier for separating early and late replicating regions is an active transcription unit oriented toward the early replicating origin. Alternatively, a barrier to replication fork movement could be mediated by a transition in chromatin structure or binding of DNA sequences to a chromosome scaffold or nuclear matrix. Further analysis of timing transitions should clarify these issues.

AUTONOMOUSLY REPLICATING SEQUENCES

One of the most difficult questions facing investigators of eucaryotic DNA replication has been whether there are specific origins of replication on chromosomal DNAs. The large size of chromosomal DNAs and the presence of multiple replication origins preclude mapping origins by the methods that have been used in procaryotic systems and eucaryotic viruses: electron microscopy and isotopic labeling. In addition, the demonstration by Harland and Laskey that *Xenopus* oocytes are able to replicate in a controlled way any DNA microinjected into them provides evidence that specific origins might not be necessary (137).

The identification of yeast chromosomal DNA sequences which have many of the properties of bacterial replication origins led to the suggestion that they function as yeast origins of replication. These autonomously replicating sequence (*ARS*) elements act in *cis* to allow the extrachromosomal maintenance of plasmids in yeasts (158, 207, 353, 357). It has recently been directly demonstrated that some *ARS* elements serve as replication origins both on plasmids (43, 163) and in the chromosome (164, 228, 325). The properties and structure of *ARS* elements as well as attempts to identify proteins that interact with them are summarized below.

Function

ARS elements were first recognized by their ability to increase the efficiency of transformation of plasmids containing them by 2 to 4 orders of magnitude compared with plasmids that transform by integration (158, 207, 353, 357). Until recently, there was no direct evidence that *ARS*s are replication origins. However, a substantial body of indirect evidence supported this hypothesis. First, genetic evidence suggested that an *ARS1*-containing plasmid was localized to the nucleus. The transmission pattern of *ARS1*-containing plasmids in crosses in which nuclear fusion was defective (*kar1*) was consistent with nuclear localization (207). In addition, *ARS* plasmid DNA is organized into chromatin whose properties are the same as bulk yeast chromatin (419). Second, like chromosomal DNA, *ARS* plasmids replicate during S phase but not in G1 phase, and they require the products of the *CDC28*, *CDC4*, *CDC7*, and *CDC8* genes for replication (107, 419). They also replicate once and only once during S phase (107). Third, the spacing between *ARS* elements has been estimated to be from 32 to 40 kb from the fraction of chromosomal restriction fragments that contain *ARS*s (22, 70). A 200-kb region of chromosome III has 11 or 12 *ARS*s spaced at an average distance of 18 kb (279). The unexpectedly high density of *ARS*s in this region is the result of two clusters of tightly spaced *ARS*s which were revealed by extensive subcloning. These estimates are broadly consistent with the estimated spacing between replication origins. Fourth, mutations in the *ARS* element of a centromere-containing plasmid increase its rate of loss, but not its rate of nondisjunction (212). Fifth, ribosomal DNA (rDNA) replication initiates preferentially at a site in the nontranscribed region of the rDNA repeat (325), and at least one rDNA repeat contains an *ARS* in the nontranscribed spacer region near the site where replication bubbles appear to originate (214, 368). Sixth, several in vitro replication systems appear to preferentially initiate replication at or near an *ARS* element, but the signal/noise ratio is low and activity in the systems is not dependent on the presence of an *ARS* element in the template DNA (65, 174, 209, 333).

While this evidence is consistent with the function of *ARS* elements as replication origins, many of the observations can be explained equally well by other hypotheses. The assay for *ARS* function, high-frequency transformation, requires not only that a plasmid replicate but also that it be stably maintained in the nucleus. Therefore, another class of explanations for *ARS* function is that they are sequences that direct plasmids to the nucleus or prevent them from being lost from the nucleus. Alternatively, *ARS* elements could serve an essential role in replication, but not at initiation. For example, they could serve an essential role in the termination of replication or decatenation of daughter molecules.

The direct evidence that at least some *ARS*s are replication origins is from two-dimensional (21) gel analysis of replicating DNA. Two different techniques have been used. In the system of Brewer and Fangman, the first dimension separates molecules on the basis of mass, and the second dimension separates on the basis of topology (46). In this system, replication intermediates form unique arcs of DNA that can be identified by hybridization with a radiolabeled probe. Restriction fragments containing internal replication bubbles form an arc that differs in shape from those carrying Y-shaped replication intermediates, allowing replication origins to be mapped. The system of Huberman et al. (163, 273) uses a first dimension that separates duplex DNA on the basis of mass and an alkaline second dimension that releases nascent strands from replication intermediates. The nascent strands form an arc that is separate from parental strands. Origins can be mapped by hybridizing blots with a series of mapped, labeled probes from a region of interest. The closer a probe is to an origin, the shorter the nascent strands it detects. The use of multiple probes allows determination of the direction of fork movement as well as positions of origins.

Both two-dimensional gel techniques have been used to show that the 2μm plasmid origin of replication is coincident with its *ARS* (46, 163). In addition, Brewer and Fangman have shown that *ARS1* functions as a replication origin on a plasmid (46). Thus, *ARS*s appear to serve as replication origins on plasmids in *S. cerevisiae*.

It is not yet clear whether all *ARS*s are capable of functioning as replication origins in chromosomes. Four chromosomal *ARS*s examined appear to function as origins in vivo. Three *ARS*s from chromosome III, A6C (164), C2G1, and J11D (S. Greenfeder and C. S. Newlon, unpublished data), as well as the rDNA *ARS* elements from chromosome XII (228), have been found to serve as chromosomal origins. However, other chromosome III *ARS* elements do not appear to function as origins in vivo. In

experiments which would have detected use of an *ARS* as an origin 20% of the time, the 40 kb of DNA at the left end of the chromosome, which contains at least four *ARS* elements, appeared to be replicated by a single fork initiated at an origin internal to the region (J. A. Huberman, personal communication). One of these, the D10B *ARS*, functions inefficiently on a plasmid (V. Van Houten and C. S. Newlon, unpublished data), so it is perhaps not surprising that it is not used as an origin. In any case, more *ARS* elements need to be examined before a general conclusion can be drawn.

Similarly, it is not yet certain whether all chromosomal origins have *ARS* function on plasmids. Two lines of evidence bear on this question. First, in earlier electron microscopic studies of 2μm DNA replication (278), as well as in studies of in vitro replication (174), a secondary origin was mapped to the small unique region of the 2μm DNA molecule. This origin was not detected in the two-dimensional gel mapping experiments (46, 163). While no satisfactory explanation for the discrepancy has been proposed, the earlier results raise the possibility that there may be, under some conditions, origins that do not function as *ARS*s.

However, studies of a 66-kb circular derivative of chromosome III suggest that *ARS*s are essential for its maintenance (279; A. Dershowitz and C. S. Newlon, unpublished data). This small ring chromosome carries two efficient *ARS*s as well as an *ARS* associated with the centromere that functions inefficiently (see next section for description of *ARS* assays). Deletion of either of the two strong *ARS*s results in a fourfold increase in the rate of loss of this chromosome, and the chromosome in which both strong *ARS*s are deleted is lost in up to half of cell divisions. Thus, there do not appear to be cryptic sequences in this 66-kb DNA segment that can replace *ARS* function. If the only essential function of *ARS*s is to act as replication origins, then this 66-kb ring has no efficient origins that are not detected as *ARS*s.

The destabilization of the 66-kb ring by deletion of a single *ARS* raises the question of how many *ARS*s are needed for efficient chromosome maintenance. If *ARS* function is required only for complete chromosome replication, then the requirement for *ARS* spacing could be determined either by the length of DNA that can be replicated from a single origin during S phase or the efficiency of initiation at *ARS*s. Based on reported fork rates (estimates are 2.4 to 6.3 kb/min at 30°C) and lengths of S phase (25 to 40 min at 30°C), 120 to 500 kb of DNA could be replicated from a single origin at which replication initiates early in S phase (186, 320). Therefore, the average spacing between origins is 3- to 14-fold shorter than is required for complete replication of DNA. Consistent with these calculations, deletion of several single *ARS*s from the full-length chromosome III has no measurable effect on its rate of loss (279). Since one of the single *ARS* deletions in the full-length chromosome leaves a longer region without an *ARS* than the deletions on the 66-kb ring chromosome, the destabilization of the ring is unlikely to result from its inability to complete replication. Instead, destabilization of the ring is more likely the result of the failure of the remaining *ARS* to initiate replication. By using two-dimensional gel techniques to map origins and evaluate their relative use, it should be possible to resolve many of these questions.

Whatever the pattern of origin usage, it is clear from density transfer experiments that chromosomal DNA replicates only once per S phase (107, 280, 417). This raises the issue of how replication is limited. Replication initiations must be prevented in regions of molecules that have already replicated, including origins of replication that have not themselves initiated but have been replicated by forks from a nearby origin. Even the 2μm plasmid amplification system does not violate this rule. Amplification appears to result from an intramolecular recombination that converts a theta-form intermediate into a double-rolling-circle intermediate that produces many tandem copies of the plasmid with a single initiation event (reviewed in reference 118). One class of explanations supposes that either old or new strands are differentially marked, e.g., by methylation. However, no evidence of DNA methylation has been found in *S. cerevisiae* (312). A second class of models suggests that replicated and unreplicated DNAs are sequestered in different compartments of the nucleus so that origins that have been replicated are not available to the replication machinery. A third class of models, exemplified by the "licensing factor" model of Blow and Laskey (36), suggests that protein binding to specific origins marks them as sites for replication initiation and that this factor has access to chromosomes only during mitosis, when the nuclear envelope breaks down or is permeable.

Properties and Assays

In contrast to integrated plasmids, *ARS*-containing plasmids are mitotically unstable and are generally lost at a rate of 0.2 to 0.3 loss per cell division (66, 87). In cultures growing under selection for an *ARS* plasmid, only 5 to 50% of cells contain plasmid; however, cells that contain plasmid have 20 to 50 copies per cell (114, 166, 418). Both the instability of *ARS*-containing plasmids and their high copy number are due to their failure to segregate properly. This was first deduced from measures of copy number and plasmid stability (418) and then directly demonstrated by pedigree analysis (267). In 30 to 60% of cell divisions, all plasmid molecules are segregated to either the mother cell or the bud, with a strong bias (19:1) in favor of the mother cell. The addition of a centromere to an *ARS*-containing plasmid provides for regular segregation and results in plasmids that are mitotically stable and are maintained at one to two copies per cell.

While the high-frequency transformation assay remains the standard for identifying *ARS*s, its use in quantitative studies of *ARS* function is limited. Transformation efficiencies are not quantitatively related to the efficiency of *ARS* function (66, 286, 356, 397). Moreover, measurements of rates of loss of these *ARS*-containing plasmids are often compromised when a plasmid integrates during the course of an experiment and is therefore stably maintained in a fraction of the population. Substantially improved assays for *ARS* function depend on the use of plasmids that carry centromeres to provide for regular segregation and copy number control and to prevent integrants (that carry dicentric chromosomes) from surviving. In addition, these plasmids carry a reporter gene to allow easy identification of cells or colonies that contain plasmid. One plasmid carries the *E. coli* β-galactosidase gene whose product can be detected in single cells through the use of a fluorescent substrate and fluorescence-activated cell sorting (348). The other two plasmids make use of the observation that blocks in two steps of the adenine biosynthetic pathway result in the production of a red pigment that stains colonies red. They carry genes that either suppress (149) or cause (212) pigment accumulation, and they have the advantage that plasmid copy number can be discerned from colony color. A recent report that detection of the red pigment is possible by

fluorescence-activated cell sorters suggests that these plasmids may also be useful for single-cell analysis (52).

By using centromere-containing vectors, quantitative measures of *ARS* function can be obtained by either determining plasmid loss rates when selection for the plasmid is relaxed (87, 120, 149, 212, 238) or measuring the fraction of cells that contain plasmid when selection for the plasmid is in force (158, 166, 353). The latter measure, called the "mitotic stability," is a function of plasmid loss rates and the number of residual divisions that a cell that has lost plasmid is able to undergo (267).

In principle, plasmid loss rates (the fraction of cell divisions in which only one of the daughter cells receives plasmid) should vary from 0 to 1. In practice, measured loss rates range from approximately 0.01 to 0.5 loss per cell division. Colonies carrying plasmids lost at a rate of about >0.25 loss per cell division grow noticeably slowly under selection compared with those with more stable plasmids, and transformants that carry plasmids lost at a rate of >0.4 to 0.5 are unable to form colonies under selective conditions (286). Naturally occurring *ARS*s exhibit the full range of efficiencies, with plasmids carrying *ARS1*, *ARS2*, and the histone H2 and H4 *ARS*s being lost at rates of 0.01 to 0.05 per cell division (149) and plasmids carrying the C2G1 *ARS* (286), the rDNA *ARS* (214), *ARS3*, and *ARS4* being lost at rates of 0.15 to 0.5 per cell division (149). In the case of strong *ARS*s, 1:0 segregations resulting from failure to replicate or plasmid loss are three to five times more frequent than 2:0 segregations resulting from nondisjunctions (149, 212). That most of the 1:0 loss events are caused by something other than failure to initiate replication is suggested by the observation that adding an extra copy of *ARS1* to an *ARS1*-containing plasmid does not improve its stability. However, plasmid stability is improved by simply increasing the size of the plasmid by inserting bacteriophage λ DNA which does not contain an *ARS* (149). Thus, measures of efficiency of strong *ARS*s are limited by the inherent instability of the plasmids currently used for *ARS* assays, and it is likely that at least some *ARS*s are much more efficient replicators than has been documented. However, despite the limitations of the assay in the high stability range, two lines of evidence suggest that intermediate to low plasmid stabilities probably do reflect increased failure to replicate. First, mutations and deletions in *ARS1* cause increases in the rate of 1:0 plasmid segregations (212, 356). Second, plasmids carrying two copies of the weak rDNA *ARS* are more stable than plasmids carrying a single copy (214).

Measurements of *ARS* efficiency need to be interpreted with some caution because the particular plasmid context in which *ARS*-containing fragments are located can affect plasmid stability. In the case of the histone H4 *ARS* (40), changing the orientation of deletion fragments changed the amount of flanking sequence required for *ARS* function (see below). In addition, the rates of loss of plasmids carrying any one of four chromosome III *ARS*s varied by two- to threefold when the fragments were inverted at either the *Bam*HI site or the *Eco*RI site of the pBR322-based vector (Van Houten and Newlon, unpublished data). In the latter study, the *ARS* fragments were large and carried several hundred base pairs of DNA on both sides of the consensus sequence (see below). Therefore, two- to threefold variations in *ARS* efficiency may not be significant when comparing different *ARS*s or even when comparing derivatives of the same *ARS* if the vector context is different.

TABLE 1. Sequenced DNA fragments containing *S. cerevisiae* chromosomal *ARS* elements[a]

ARS	Reference(s)
ARS1	378
ARS2	379
HML E	109
HML I	109
HMR E	1
HMR I	1
HO	195, 196, 322
Histone H4	40, 41
C2G1	286, 287
A6C	287
J11D	287
H9G	287
pY20	110
2μm	49, 50

[a] Includes only those *ARS* elements for which >100 bp of DNA sequence is available. Short segments of DNA sequence have also been reported for *ARS3* (352), *ARS121*, *ARS137*, *ARS245*, *ARS131B*, and *ARS131S* (341).

Structure-Function Analysis

Definition of the specific sequences required for *ARS* function is important for the identification of potential protein recognition sequences or sequences that act in other ways to mediate *ARS* function. With the exception of *ARS*s located within repeated telomeric elements (70, 71), *ARS*-containing DNA fragments do not have sufficient regions of homology to cross-hybridize. Nevertheless, comparisons of DNA sequences of *ARS*-containing fragments have led to two generalizations: *ARS* elements have a significantly higher A+T content (73 to 82%) than chromosomal DNA (60%), and all *ARS* elements contain one or more copies of at least 10 of 11 nucleotides of the core consensus sequence 5′-(A/T)TTTAT(A/G)TTT(A/T)-3′ (50, 352), which is also called domain A (66). This is true for *S. cerevisiae* chromosomal *ARS*s, the 2μm plasmid *ARS*, and DNA fragments from other organisms that have *ARS* activity in *S. cerevisiae*. Sequenced fragments containing *S. cerevisiae ARS* elements are listed in Table 1.

The sequences required for the function of four *S. cerevisiae* chromosomal *ARS*s have been studied in detail. In each case, deletions define a small region of 25 to 65 base pairs (bp) that is essential for *ARS* function, and, in the two cases in which it has been studied, deletions in flanking regions 100 to 300 bp from the core consensus sequence reduce function as measured by plasmid stability. The essential region includes a core consensus sequence, two to three nucleotides 5′ and a variable number of nucleotides 3′ to the T-rich strand of the core consensus. Point mutations and small deletions and small substitutions within the consensus sequence and the two or three nucleotides that immediately flank it abolish function (40, 41, 66, 195, 196, 286, 348, 356). Two additional results demonstrate that a core consensus sequence alone is not sufficient for *ARS* function. Small fragments containing an exact match to the consensus sequence do not have *ARS* activity in plasmids (66, 286), and the consensus sequence occurs in yeast DNA fragments that do not have *ARS* function (41, 286).

Although the *ARS* consensus sequence was first identified more than 5 years ago (50, 352), DNA sequence comparisons of *ARS*-containing fragments have not allowed a quick definition of highly conserved nucleotides. This is because most such fragments have more than one sequence with at least 90% homology to the 11-bp consensus sequence.

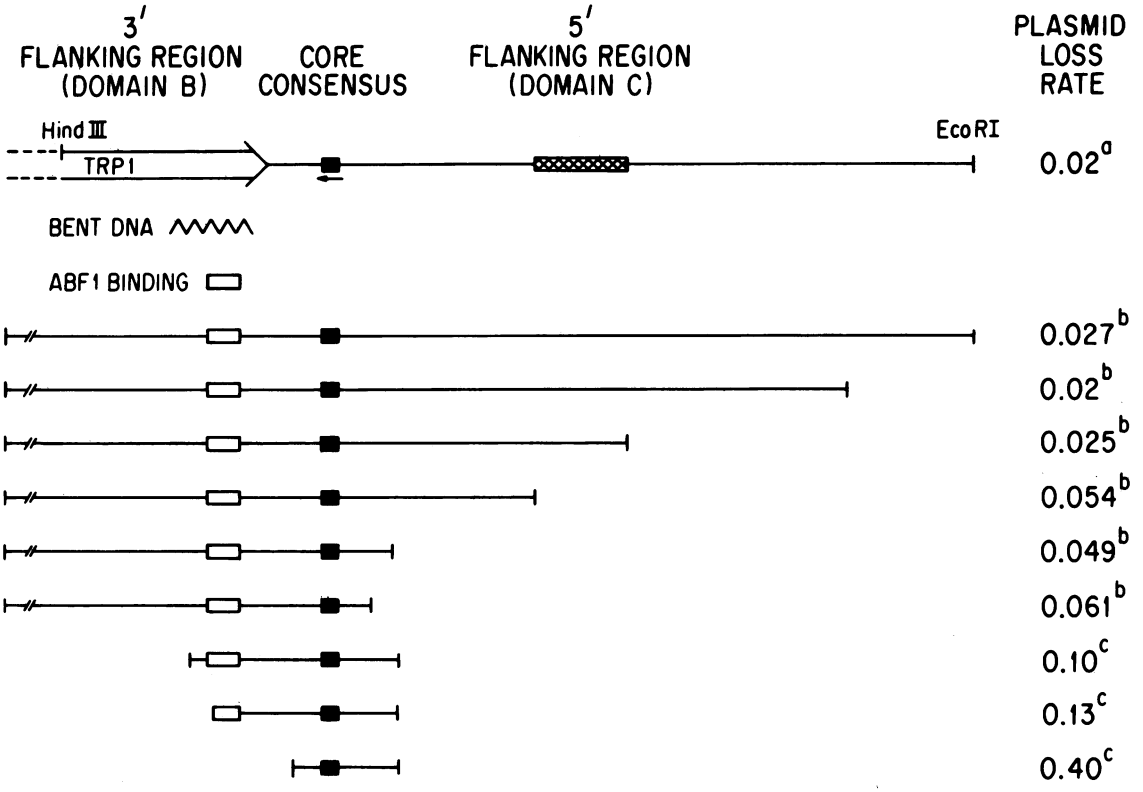

FIG. 1. Structure of *ARS1*. The top line represents the 837-bp *Hin*dIII-*Eco*RI fragment from chromosome IV that contains *ARS1*. The 3′ end of the *TRP1* open reading frame is shown by an open arrow. A region of bent DNA (6, 345) and an ABF1 (*ARS* binding factor I) binding site (53, 92, 337) near the 3′ end of the *TRP1* open reading frame are indicated. The exact match to the *ARS* core consensus sequence is indicated by a solid rectangle. The T-rich strand of this sequence is in the lower strand, so its 5′-flanking regions are to the right and 3′-flanking regions are to the left. The cross-hatched box indicates the region of the 5′-flanking sequences that contributes to *ARS* function. Rates of loss of the YRp14-*CEN4* plasmid (149) containing the indicated fragments are shown. This plasmid contains a weak *ARS*, *ARS3*, and is lost at a rate of >0.4 loss per cell division. Data are from (a) Hieter et al. (149), (b) Strich et al. (356), and (c) Diffley and Stillman (92). While others have reported similar results (see text), these were chosen because flanking-vector sequences were conserved and the loss rates were measured in the same vector.

Therefore, detailed deletion analysis is required for definition of the essential core consensus sequence and the number of examples is limited. Thus, while at least some sequences with 90% homology to the consensus sequence can provide the essential function (195, 196, 287), the required nucleotides have not yet been identified.

From the data presently available, it is difficult to make a definitive statement about the size of the smallest fragment with *ARS* function because adjacent vector sequences can affect function and because in many cases the minimum core sequence defined by deletion analysis has not been tested for activity in the absence of flanking sequences. In a study in which the histone H4 *ARS*-containing fragment was examined in both orientations relative to the vector, the 3′-flanking-sequence requirement changed from 57 to 67 bp to 12 to 29 bp with the change in orientation (40). A 19-bp fragment containing the core consensus sequence of *ARS1* has *ARS* activity in a centromere vector (348), but not in a somewhat different context in an integrating vector (66). Moreover, a 100-bp fragment that included the 19-bp fragment did not have *ARS* activity in a different centromere vector (93). It is not yet clear whether flanking-vector sequences act as positive or negative effectors of *ARS* activity. In the histone H4 *ARS*, linker scanning substitutions were constructed through the entire 75-bp region required for *ARS* function (40). The only mutations that affected function (high-frequency transformation in this case) were in or near the exact match to the core consensus. It may be that some physical property of the flanking region and not specific sequences is required for function. Alternatively, the important sequences may be present in multiple copies.

The two *ARS*s in which deletions have been examined for efficiency of function by measuring plasmid loss rates give somewhat different pictures of the sequences required for full function. In *ARS1*, sequences on both sides of the core consensus contribute to function (Fig. 1). The contribution of sequences 5′ to the T-rich strand of the core consensus (domain C) can be seen in integrating vectors only when part of the 3′-flanking sequence (domain B) is deleted (66). In centromere-containing vectors, the presence of 5′-flanking sequences improves plasmid stability two- to threefold (212, 348, 356). The sequences in the 5′-flanking region that contribute to *ARS* function have been mapped by linker insertions and deletions to an 80-bp region between 200 and 280 bp 5′ to the core consensus (212, 356). This region is interesting because it coincides with the replication origin used in vitro (65).

The 3′-flanking region of *ARS1* has not been examined as systematically as the 5′-flanking region. Deletions of the 3′-flanking sequences have a more profound effect on *ARS* function than deletion of 5′-flanking sequences. Deletions that remove all of the 3′-flanking sequences cause at least a 10-fold increase in plasmid loss rates (348, 352, 356) and, in

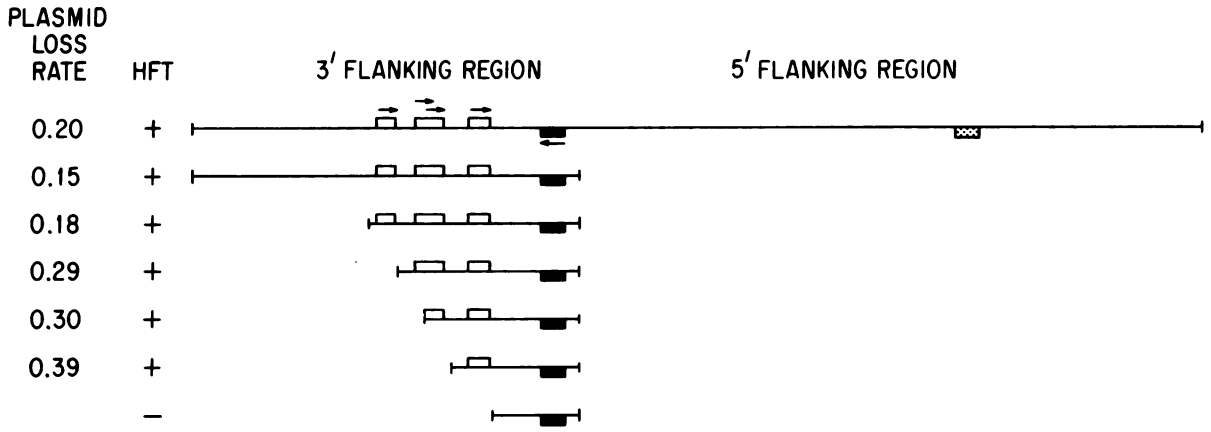

FIG. 2. Structure of the C2G1 *ARS*. The 522-bp *Eco*RI-*Cla*I fragment from chromosome III that contains the C2G1 *ARS* is shown on the top line. The perfect match to the *ARS* core consensus sequence is represented by the solid box below the line. As in *ARS1*, the T-rich strand of this sequence is in the lower strand. The open boxes represent 9 of 11 matches to the core consensus sequence in the upper strand, and the stippled box is a 10-of-11 match to the core consensus sequence in the lower strand. Deletion fragments were assayed in the *Bam*HI site of pVHA, which is similar to YRp14-*CEN4* except that *ARS3* was deleted. Plasmid loss rates are in loss events per cell division. Data are from reference 286. HFT, High-frequency transformation.

some vector contexts, eliminate *ARS* function (93, 348). Effects of deletions on plasmid stability can be seen for deletions that leave about 90 bp of the 3'-flanking region intact (345), and a very large decrease in plasmid stability is associated with a deletion that removes an additional 60 bp (93, 348, 356). Insertions in the 3'-flanking region also reduce plasmid stability (93, 356, 397), suggesting that the spatial relationship between these sequences and the core consensus sequence is important.

In summary, full function of *ARS1* requires an exact match to the core consensus sequence as well as flanking sequences on each side. Because *ARS* activity can be detected in plasmids containing the core consensus and either 5'- or 3'-flanking sequences, it may be that these sequences contain redundant information. However, these results must be interpreted with some caution. It is clear that 3'-flanking sequences have a larger influence than 5'-flanking sequences on *ARS* activity, and in some vector contexts a portion of the 3'-flanking sequence is essential for detectable *ARS* function.

In contrast to *ARS1*, the 5'-flanking sequences of the chromosome III *ARS*, C2G1, have a negative rather than a positive effect on *ARS* function (286). As in the case of other *ARS*s, the exact match to the core consensus sequence is essential for function. In the 3'-flanking region (domain B), sequences 90 bp from the core consensus sequence contribute to *ARS* function and between 35 and 45 bp of flanking sequence is required for function. The loss of *ARS* function is gradual with progressive deletion of 3'-flanking sequences, and significant increases in plasmid loss rates are correlated with deletion of sequences with 80% homology to the core consensus that occur in domain B (Fig. 2). Small internal deletions within domain B are also consistent with a role for the near matches to the core consensus in *ARS* function. As in the case of *ARS1*, insertions into domain B of the C2G1 *ARS* reduce function (287). Most other *ARS* sequences examined contain multiple near matches to the core consensus in domain B, and the frequency with which these near matches occur in *ARS*s is much higher than expected in a sequence of the A+T content of the *ARS*s analyzed. Therefore, it may be that domain B function is mediated by near matches to the core consensus. By analogy with a number of procaryotic replication origins and the simian virus 40 (SV40) replication origin that contain multiple binding sites for a replication initiator protein, it is possible that the core consensus sequence defines a binding site for a yeast initiator protein.

The C2G1 *ARS* is about threefold less efficient than *ARS1*, and its activity is similar to deletions of *ARS1* that lack 5'-flanking sequences. This raises the possibility that the difference between the two *ARS*s is that 5'-flanking sequences contribute to *ARS1* function. It will be of interest to determine which organization is more typical of chromosomal *ARS*s in general and whether the 5'-flanking domain of *ARS1* can improve the efficiency of the C2G1 *ARS*.

A fifth chromosomal *ARS* that has been studied in some detail and which has a number of interesting properties is the *HMR E ARS*. It is within and is a required part of the regulatory region (the *HMR* silencer) that prevents expression of the silent mating-type locus, *HMR*, on the right arm of chromosome III (1, 43, 206). This *ARS* is unique because it enables plasmids to segregate better than other *ARS*-containing plasmids studied. The ability of the *HMR E ARS* to direct segregation requires the products of the *trans*-acting genes (*SIR1* to *SIR4*) that are required for transcriptional repression of *HMR*. Plasmids carrying both the *HMR E ARS* and a centromere are less stable than plasmids carrying either element alone, demonstrating that the segregation functions provided by the two elements are antagonistic in plasmids (206). Detailed analysis of this region has been complicated by the recent finding that there are two separable *ARS* elements within a 2.95-kb restriction fragment that carries *HMR E* (43). However, it is now clear that, for the *ARS* required for silencer function, DNA fragments carrying the core consensus sequence and its 3'-flanking region have *ARS* activity. The segregation function requires protein-binding sites in the 5'-flanking region of this *ARS* (43, 205).

Deletion analysis of the C2G1 *ARS* suggests that the required 3'-flanking sequences are copies of the core consensus sequence. While this hypothesis can account for the available data from other *ARS*s, there are other possibilities that merit consideration. The first class of explanations includes those that emphasize the physical properties of a DNA sequence. For example, in *oriC*, bacteriophage λ, and SV40, origin function requires an A+T-rich segment of DNA

adjacent to the initiator protein-binding sites where a localized unwinding of the DNA duplex occurs and the rest of the replication complex is assembled. Binding of the λ initiator protein, the O protein, induces unwinding in a supercoiled molecule (329), and the SV40 initiator protein, T antigen, is a helicase (96, 349). At *oriC*, binding of the initiator, dnaA protein, or the recruitment of a helicase by protein-protein interaction with dnaA protein is thought to catalyze the unwinding (11, 334).

It was first proposed by Broach et al. that the high A+T content of *ARS*s contributes to function by facilitating DNA unwinding (50). The unwinding potential of *S. cerevisiae ARS* sequences has been analyzed by measuring the sensitivity of *ARS* regions in purified supercoiled plasmids to mung bean nuclease (384, 385). This nuclease cleaves DNA in non-B conformations, including single-stranded DNA in unwound regions. The two *ARS* elements that have been studied unwind in preference to other plasmid DNA. In addition, the unwinding capacity of several progressive external deletions of the histone H4 *ARS* correlates with *ARS* function as measured by high-frequency transformation. Furthermore, a nonfunctional deletion could be rescued by insertion of an easily unwound pBR322 sequence.

In an attempt to distinguish between the roles of core consensus sequence elements and A+T content in mediating flanking sequence function, Palzkill and Newlon used oligonucleotides containing a consensus sequence but with an A+T content of only 60% to construct synthetic *ARS*s (286). Two consensus sequences, in either direct or inverted orientation, were sufficient for high-frequency transformation. However, efficiency of function was critically dependent on both number and orientation of consensus sequences. The synthetic *ARS* that is as efficient as the C2G1 *ARS* is organized with four copies in tandem 3′ to and inverted with respect to a single copy of the consensus (Fig. 2). The orientation dependence of this construct suggests that specific sequences are important for function. In addition, the construct demonstrates that a fully functional *ARS* need not have an A+T content that is higher than the average chromosomal DNA A+T content. Since the A+T-rich pBR322 fragment used to rescue function of the histone H4 *ARS* deletion (385) contains two 9-of-11 matches to the core consensus sequence, it is possible that the rescue of function was from the addition of specific sequences rather than simply the addition of easily unwound DNA. By using synthetic constructs for measurements of efficiency of function and unwinding capacity, it should now be possible to test directly the relative importance of both specific sequences and ease of unwinding for *ARS* function.

Another structural feature common to the λ and SV40 replication origins is the presence of bent DNA (323, 412). The 3′-flanking region of *ARS1* also contains a segment of bent DNA about 80 bp from the core consensus (6, 345). Deletion of this region, which coincides with a protein-binding site (see below), has a small but detectable effect on *ARS* function that can be magnified by growth on galactose (93, 345). Furthermore, efficient *ARS* function can be partially rescued by inserting synthetic bent DNA in an *ARS1* deletion lacking 3′-flanking DNA (397). Other *ARS*s contain tracts of A and T that might be expected to form a bend, but they have not been analyzed directly (100). However, the C2G1 *ARS* does not contain bent DNA (T. Palzkill, Ph.D. thesis, University of Iowa, Iowa City, 1988). Thus, no requirement for bent DNA in *ARS* function is established.

The second class of explanations for the function of the 3′-flanking region includes those which posit the binding of a specific protein, either to mediate function or to protect the *ARS* element from disturbance, for example, by transcription through it (345). In an attempt to identify additional potential protein-binding sites, Palzkill et al. found a second 11-bp consensus sequence (the 3′-conserved sequence) present in the 3′-flanking region of many but not all *ARS*s (287). However, small internal deletions and linker insertions that remove this sequence from the C2G1 *ARS* have no effect on *ARS* function as measured by plasmid stability (286).

A potential need for transcription terminators adjacent to *ARS* elements is suggested by their juxtaposition to transcription units in chromosomes. Of the 14 *ARS* elements mapped with respect to adjacent transcription units, all but 1 are located within 100 bp 3′ to an open reading frame and are thus, presumably, preceded by transcription terminators (compiled in reference 276). The exceptional *ARS* is located within the coding sequence of the *HO* gene (322). Plasmid constructs that place an inducible *GAL* promoter on either side of *ARS1* become unstable when the promoter is induced. Moreover, when DNA fragments containing transcriptional terminators were placed downstream of the promoter, the plasmids retained stability (346). Although transcription through *ARS1* itself was not detected in these experiments, the results do suggest that transcription that impinges on an *ARS* may impair its function.

trans-Acting Factors

The most likely function for the *ARS* core consensus sequence is to serve as a protein-binding site. Analysis of chromatin structure in the *ARS1* region has shown that the functional *ARS* is within a nuclease and methidium propylethylenediaminetetraacetic acid · Fe(II) hypersensitive region that is bounded by phased nucleosomes in both plasmids (231, 373) and in the *ARS1* region of chromosome IV (230). A deoxyribonuclease I footprinting analysis of the chromosomal hypersensitive region also provided direct evidence for localized protein-DNA contacts within the region (230). The most prominent protein contact is over the core consensus sequence. There is also evidence for additional protein contacts in both flanking regions. Thus, in vivo, *ARS1* appears to be bound by proteins other than histones, and there is evidence that the core consensus sequence is a protein-binding site.

Both biochemical and genetic approaches have been taken to identify proteins that interact with *ARS*s. Gel retardation assays and DNA filter-binding assays have been used to identify at least three proteins that bind to *ARS*-containing DNA. Shore et al. (337) found a factor, SBF-B, that binds to the *HMR E ARS* and the 3′-flanking region of *ARS1*. This factor protects specific regions of these two *ARS*s from deoxyribonuclease I digestion and is probably the same as the ABFI (*ARS* binding factor I) protein characterized in three other laboratories (53, 92; K. S. Sweder, P. Rhode, and J. L. Campbell, J. Biol. Chem., in press). The binding site associated with *ARS1* is within the C-terminal region of the *TRP1* open reading frame (378). In addition to *ARS1* and the *HMR E ARS*, ABFI binds to sites associated with the *HMR I*, *HML I*, and 2μm *ARS*s and with *ARS2*. It also binds to the region between the *HIS3* and *DED1* genes, which is several kilobases from any *ARS* element. It failed to bind to several other *ARS*s tested, including the *ARS*s associated with *HML E* and histones H2B (*TRT3*) and H4. Although individual ABFI binding sites are variable in sequence, a consensus binding sequence with dyad symmetry has been

deduced. The ABFI protein is relatively abundant (500 molecules per cell) and has a molecular weight of 135,000 (53). The role of ABFI in *ARS* function appears to be minimal since deletion of its binding site from *ARS1* results in only a small decrease in plasmid stability when assayed under standard conditions in glucose-containing medium (92, 345). Its role in *HMR E ARS* function is unclear. It has been reported that deletion of the ABFI binding site reduces plasmid stability (43), but it has not been determined whether the replication or segregation function of the *HMR E ARS* is affected. In addition, a point mutation in the ABFI binding site that abolishes ABFI binding in vitro does not affect plasmid stability (205).

The second *ARS*-binding protein, called OBF1, is similar to ABFI in the sense that it binds to a subset of *ARS*s. This protein was identified on the basis of its binding to a fragment containing *ARS120* from a telomeric X region (102, 103). OBF1 binding protects a 26-bp sequence approximately 200 bp 3′ to the core consensus sequence of *ARS120*. On the basis of competition experiments, OBF1 also appears to bind sequences in other *ARS*s from telomeric X regions and in *ARS121*, a single-copy *ARS* element. However, it does not bind sequences in *ARS1*, the *HMR E* and *HML E ARS*s, or *ARS*s from telomeric Y′ regions. As in the case of ABFI, deletion of the OBF1 binding site from *ARS120* has a negligible effect on plasmid stability.

The third *ARS* binding protein, ABFII, does not bind specifically to *ARS* elements, but binding of several molecules of this protein to *ARS1* induces bending in the DNA that is not seen when ABFII binds to pBR322 sequences (93).

Conclusions about the roles of any of these proteins in *ARS* function await further studies. Although ABFI and OBF1 are dispensible for *ARS* function on plasmids, it is possible that they affect a function that has not been assayed on plasmids, for example, timing of replication. Alternatively, they could perform a function in the chromosome that is not required on plasmids. A third possibility is that they are not required for *ARS* function and that their binding to *ARS*-containing DNA is fortuitous. For example, ABFI binding sites can function as upstream activating sequence elements in plasmid assays (43, 53). It has also been proposed that ABFI may function in transcription termination (53, 346). The reason that assays for DNA-binding proteins have not yet succeeded in identifying a core consensus sequence binding protein is not clear.

Another approach to identifying genes involved in *ARS* function is to screen for mutations that affect the stability of *ARS*-containing plasmids. Mutants unable to stably maintain either centromere-containing *ARS* plasmids (124, 238) or the endogenous 2μm plasmid (203) have been identified. For centromere-containing plasmids, 43 mutations that define 18 complementation groups were isolated. Five of these *MCM* (for minichromosome maintenance) genes have mutant alleles that affect specific *ARS*s, while all mutations in the remaining genes have similar effects on plasmids containing the 20 *ARS*s tested. The *mcm* mutations might be expected to identify genes whose products have either direct or indirect effects on plasmid stability. Those that affect specific *ARS*s are more likely than the others to encode proteins that interact directly with *ARS* elements. However, since all of the mutations that show *ARS* specificity affect the same subset of *ARS*s, even they may be in genes whose products have indirect effects on plasmid maintenance.

Three *mcm* mutants that have an *ARS*-specific phenotype have been further characterized. In strains carrying the *mcm2-1* mutation, circular and linear plasmids as well as full-length chromosomes are lost at higher than normal rates. The observation that the loss events reflect simple losses (1:0 segregations) rather than nondisjunctions is consistent with the mutation affecting replication (342). In addition, this mutation stimulates mitotic recombination, a property associated with many DNA replication defects (142). *mcm2* mutants grow more slowly at 36°C than wild-type strains. The *MCM2* gene has been cloned by complementation of the conditional growth phenotype of the *mcm2* mutant (124).

Mutations in the *MCM1* and *MCM3* genes are also pleiotropic: *mcm1* mutants are *MATα*-specific steriles, and the *mcm3* mutant is temperature sensitive for growth. These genes have also been cloned by complementation of the sterile phenotype or the conditional growth phenotype. The sequence of the *MCM1* gene contains an open reading frame that is predicted to encode a 143-amino-acid protein; disruption of this open reading frame is lethal (124, 341).

Using a similar approach, Kikuchi and Toh-e identified mutations that result in the instability of the endogenous 2μm plasmid (203). The seven mutations analyzed define one locus, designated *MAP1*. *ARS1*-containing plasmids are also unstable in these mutants, and plasmids containing more than one *ARS* are more stable, suggesting that the mutations affect *ARS* function. The *mcm2-1* mutation is the only *mcm* mutation that affects the maintenance of the 2μm plasmid; whether mutations at the *MAP1* locus are allelic to mutations at the *MCM2* locus has not been determined.

Kearsey and Edwards have used the alternative approach of identifying mutations that increase the mitotic stability of plasmids carrying weak *ARS* elements (198). Thirteen mutants, designated Rar$^-$, were studied. Most were recessive and nine that were tested defined six complementation groups. The *rar1-1* mutation also conferred temperature-sensitive growth. This phenotype was used as the basis for cloning the gene by complementation. The predicted open reading frame encodes a protein of 443 amino acids that has no significant homology to other proteins in the protein data bank.

Whether any of these genes encodes a protein that interacts directly with *ARS* sequences remains to be determined. By analogy with mutants identified by their inability to maintain a double-stranded RNA killer plasmid in *S. cerevisiae* (*mak* mutants), many of these mutants might be expected to have indirect effects on plasmid stability. For example, *MAK1* encodes topoisomerase I and *MAK8* encodes ribosomal protein L3 (reviewed in reference 396), and it is not obvious that either of these proteins should have direct effects on the maintenance of a double-stranded RNA.

Heterologous *ARS* Elements

DNA from a large number of organisms has been tested for *ARS* function in *S. cerevisiae*, and a subset of chromosomal DNA fragments from all eucaryotes examined has *ARS* activity (Table 2). In addition, a number of extranuclear DNAs from cytoplasmic organelles and viruslike particles contain sequences that function as *ARS* elements (Table 2). In contrast, no *E. coli* DNA sequence has been found to have *ARS* activity (354). The only report of a procaryotic DNA segment with *ARS* function in *S. cerevisiae* is from a *Staphylococcus aureus* plasmid (130). Insofar as it has been examined, all of the heterologous *ARS* elements contain at least one copy of the core consensus sequence (4, 5, 195, 204, 245, 260, 374, 386). It has been suggested that an additional short consensus sequence contributes to *ARS*

TABLE 2. Heterologous DNA fragments with *ARS* activity in *S. cerevisiae*

Fragment	Reference(s)
Eukaryotic chromosomal DNA	
Schizosaccharomyces pombe	247
Candida maltosa	194
Neurospora crassa	354
Ustilago maydis	13
Dictyostelum discoideum	354
Physarum polycephalum	127
Chlamydomonas reinhardii	232
Caenorhabditis elegans	354
Drosophila melanogaster	244, 354
Lytechinus variegatus	39
Xenopus laevis	195
Mouse	321
Human	260
Zea mays	354
Extrachromosomal DNA plasmids	
S. cerevisiae 2μm	49
Kluyveromyces lactis killer plasmid	374
Staphlococcus aureus plasmid	130
Tetrahymena thermophila rDNA	208
Mitochondrial DNA	
Saccharomyces cerevisiae	32, 166, 167
Podospora anserina	225
Crithidia fasciculata	204
Paramecium aurelia	224
Xenopus laevis	414
Chloroplast DNA	
Chlamydomonas reinhardii	232, 386

function of *Drosophila* sequences, but the deletion data presented are not adequate to test the hypothesis (245).

It is becoming clear that heterologous sequences that have *ARS* function in *Saccharomyces* spp. are not the same sequences that have *ARS* activity in organisms from which they were isolated. There are fragments of *Schizosaccharomyces pombe* chromosomal DNA that have *ARS* activity in both *S. cerevisiae* and *Schizosaccharomyces pombe* (247). However, many fragments that have *ARS* function in *Schizosaccharomyces pombe* do not function in *S. cerevisiae* and vice versa (21, 180, 247). Similarly, although they reside in the same restriction fragment, sequences necessary for *ARS* function in *S. cerevisiae* are different from the sequences required for origin function in the *Tetrahymena* rDNA molecule (5), and sequences required for *Kluyveromyces lactis* killer plasmid replication are separable from the *ARS* active in *S. cerevisiae* (374). Finally, mouse sequences with *ARS* function in *S. cerevisiae* do not replicate autonomously in mouse cells (321). Thus, *ARS* function in *S. cerevisiae* is not a useful indicator of autonomous replication in other species.

PROTEINS REQUIRED FOR REPLICATION

Progress in understanding the molecular details of DNA replication in the well-understood procaryotic systems has depended on the isolation and characterization of replication origins and mutants defective in DNA replication, as well as on the development of in vitro assays for individual proteins and of in vitro replication systems. For plasmids containing the *E. coli* replication origin *oriC* and for those containing the bacteriophage λ replication origin, there are now in vitro systems composed of purified proteins that faithfully initiate replication (for a recent review, see references 10 and 254). In addition, there are purified proteins from *E. coli* (reviewed in reference 10), bacteriophage T4 (reviewed in reference 68), and bacteriophage T7 (reviewed in reference 161) that carry out faithful leading- and lagging-strand synthesis on double-stranded templates. The replication of an *oriC*-containing plasmid requires at least 11 enzyme complexes which are encoded by more than 20 genes.

Initiation of replication at *oriC* and *oriλ* requires the binding of an initiator protein and its recruitment, through protein-protein interactions, of other proteins into a complex called the replisome. The earliest known event is the unwinding of the origin region, first by the initiator protein and then by its recruitment of a helicase. The primer for the initiation of replication is probably made by DNA primase, the protein that functions in laying down primers for lagging-strand synthesis. The ColE1 plasmid uses a different mechanism for initiating DNA replication. In this case, an RNA transcript is initiated at a promoter upstream of the origin and is processed by ribonuclease H to form the primer for leading-strand synthesis (96).

Synthesis of daughter strands in these systems requires the participation of a number of proteins, including a helicase to unwind the duplex DNA at the replication fork, single-strand binding protein to coat the single strands, DNA polymerase III (an enzyme with seven different subunits) for synthesis of the daughter strands, DNA primase for synthesis of primers, ribonuclease H for primer removal, and DNA ligase for ligating together Okazaki fragments on the lagging strand. In addition, DNA topoisomerases are required to release the supercoiling induced by unwinding the parental duplex and for decatenation of daughter molecules. It is possible that DNA polymerase I also functions in some aspect of DNA replication, possibly in replacement of RNA primers.

It is likely that eucaryotic DNA replication requires the participation of a similar collection of proteins. The identification of proteins required for in vitro SV40 replication (226, 227) has revealed the requirement for a number of anticipated enzyme activities. The initiator protein is the SV40-encoded T antigen which has helicase activity and has been shown to unwind the origin (90, 96, 349). A cellular protein that has not yet been purified is required with T antigen to form an active presynthesis complex (106, 405). The in vitro system also requires DNA polymerase α (253), topoisomerases I and II (409), a single-strand DNA-binding protein (409), and a factor that stimulates DNA polymerase δ, proliferating cell nuclear antigen (309).

Two approaches are being used to identify mutations in genes whose products are required for DNA replication. The first is the classical approach of screening collections of conditional (temperature-sensitive or cold-sensitive) mutants for replication defects. The second approach, "reverse genetics," makes use of a purified protein of interest to develop reagents (either antibodies or oligonucleotide probes) for cloning the gene. The cloned gene can then be disrupted or mutagenized in vitro and reintroduced into the genome for an assessment of the mutant phenotype. This approach is laborious and can lead to disappointing results if the purified protein is not actually required for the process of interest.

Cell Division Cycle Mutants

Among the large collection of temperature-sensitive mutants in *S. cerevisiae* is a class of mutants that arrest at a

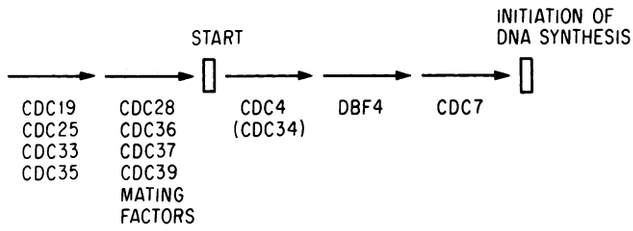

FIG. 3. Pathway leading to initiation of DNA synthesis. In this diagram, each arrow represents a step whose completion is dependent on the prior completion of the previous step. Evidence for these steps is based largely on reciprocal shift experiments. Gene products listed below a single arrow all appear to be required for that step. *CDC34* is in parentheses because its order with respect to *CDC4* has not been established. *cdc34* mutants have the same terminal phenotype as *cdc4* mutants (reviewed in reference 310). DBF4 is placed on a separate step because it appears to block after *cdc4* but before *cdc7* (184).

specific stage in the cell cycle when shifted to the nonpermissive temperature. About 10% of temperature-sensitive and cold-sensitive lethal mutations are in *CDC* (for cell division cycle) genes that define more than 50 complementation groups (141, 259, 310). These genes encode products required for specific steps in the cell cycle. By ordering the requirements for mutant gene products with respect to the action of other agents that arrest the cell cycle or with respect to the requirement for other *CDC* gene products, the steps have been placed in a series of dependent pathways whose completion is necessary for cell division (reviewed in reference 310). The mutations in the pathway that leads from "start" to DNA synthesis and nuclear division identify genes that may be involved in aspects of DNA replication.

Mutants blocked in G1. The pathway leading to the initiation of DNA synthesis is diagrammed in Fig. 3. Strains carrying mutations in any of these genes fail to initiate DNA synthesis at the nonpermissive temperature (138, 139, 147, 184, 298, 313). The first two steps define the major control point in the cell cycle, termed start, at which mating pheromones and nutrient limitation arrest the cell cycle. Completion of start is signalled by spindle pole body duplication and bud emergence. The next step requires the action of the *CDC4* and *CDC34* gene products and is signalled by separation of the spindle pole bodies (reviewed in reference 310). Finally, the action of the *CDC7* and possibly the *DBF4* gene products is required for the initiation of DNA synthesis. In contrast to *dbf4* mutants, *cdc7* mutants can complete the protein synthesis required for DNA replication while blocked at the nonpermissive temperature, suggesting that the *cdc7* block is closer to the initiation of DNA synthesis (147, 184). However, the *CDC7* gene product is not required for premeiotic DNA replication (328); therefore, if the *CDC7* protein is directly involved in the initiation of mitotic DNA replication, then mitotic and premeiotic DNA replication must have different requirements.

Recent molecular analyses of the products of several *CDC* genes suggests that progression through the G1 phase of the yeast cell cycle and possibly the initiation of DNA replication may be regulated by protein modification. *cdc35* mutations are allelic to *cyr1* mutations which define the structural gene for adenylate cyclase (reviewed in reference 246). The *CDC28* gene product is a protein kinase (314). The predicted products of the *CDC36* and *CDC4* genes are homologous to each other and to the oncogene *ets* (296, 411). In addition, the *CDC4* product shows homology to the G protein transducin (115, 411). *CDC34* encodes a ubiquitin-conjugating enzyme that utilizes histones H2A and H2B as substrates in vitro (126). Both *CDC4* and *CDC34* gene products may play a role in histone metabolism since transcription of the histone H2A and H2B gene pairs does not occur until after the *cdc4*-sensitive step (395). Finally, the predicted *CDC7* gene product shows homology to protein kinases (291). Interestingly, a protein kinase activity that is temperature sensitive in *cdc7* strains has been found in a multiprotein replicative complex that is capable of catalyzing DNA replication in vitro (172).

Mutants blocked in S phase and nuclear division. The properties of mutations in the 21 genes that define the remainder of the DNA synthesis and nuclear division pathway are summarized in Table 3. All of these mutants arrest as cells with large buds and a single nucleus that in some cases appears to be dividing (reviewed in reference 310). Temperature-sensitive mutations in genes that are required for DNA chain elongation might be expected to result in immediate cessation of DNA synthesis after a shift to the nonpermissive temperature. Among the original collection of cell cycle mutants, only *cdc8* and *cdc21* mutants showed the "immediate shutoff" phenotype (138, 139). These genes are now known to function in precursor supply. *CDC8* is the structural gene for thymidylate kinase (190, 331) and *CDC21* encodes thymidylate synthase (28, 121). Mutations that result in the same immediate shutoff of DNA synthesis phenotype have been identified in two other genes, *DBF1* and *DBF2*, but their gene products have not been identified (183). DNA synthesis in *cdc8* mutants is temperature sensitive in vitro (146, 173, 209, 216), raising the possibility that the *CDC8* gene product plays a role in replication in addition to its role in precursor supply. The finding that the herpesvirus thymidine kinase gene (which encodes an enzyme having both thymidine kinase and thymidylate kinase activities) can complement *cdc8* mutations (331) suggests that, if the *CDC8* gene product has a second role, the mutant *cdc8* gene product is still capable of carrying out this function. It would be of interest to determine whether the herpesvirus thymidine kinase gene can complement a *cdc8* deletion. Alternatively, the *CDC8* gene product may be required simply as a component of a deoxythymidine triphosphate regenerating system in vitro.

Although they do not cease DNA synthesis immediately at the nonpermissive temperature, at least half of the remaining cell cycle mutants that are blocked in S phase or nuclear division encode products that are likely to be required for some aspect of DNA replication. The requirement for many of the gene products has been ordered with respect to the hydroxyurea-sensitive step (140, 193, 258). Hydroxyurea inhibits DNA synthesis in yeasts (343), presumably by inhibiting ribonucleotide reductase (233, 388). Strains carrying mutations in *CDC2*, *CDC9*, and *CDC40* are partially or completely sensitive to hydroxyurea after incubation at the nonpermissive temperature, suggesting that the DNA synthesis at high temperature is incomplete or defective. In addition, the *CDC6* function appears to be a prerequisite for the hydroxyurea-sensitive step even through *cdc6* mutants synthesize DNA at the nonpermissive temperature. The *CDC9* gene product is DNA ligase (15, 16, 121, 182). The partial hydroxyurea sensitivity of *cdc9* mutants suggests that single-stranded gaps that require additional synthesis for repair may accumulate at high temperature. Further studies of *cdc2* mutants revealed that they fail to replicate approximately one-third of their DNA at the nonpermissive temperature (83) and that DNA synthesis in permeabilized cells is

TABLE 3. Genes required for DNA synthesis and nuclear division

Gene	Product	Function[a]	Remarks and reference(s)
CDC2	?	iDS or DS	Replicates about two-thirds of genome at 36°C (83); remains sensitive to hydroxyurea after incubation at 36°C (140); permeabilized cells temp sensitive for DNA synthesis (218); increased rates of chromosome loss and mitotic recombination at maximum permissive temp (142); heterogeneous with respect to MBC[b] sensitivity (406)
CDC6	?	iDS or DS	Synthesizes DNA at 36°C, but is required prior to hydroxyurea-sensitive step (140); heterogeneous with respect to MBC sensitivity after incubation at 36°C (406); increased rates of chromosome loss and mitotic recombination at maximum permissive temp (142)
CDC8	Thymidylate kinase	PS	Ceases DNA synthesis quickly after temp shift (139); permeabilized cells temp sensitive for DNA synthesis (146, 216, 218); structural gene for thymidylate kinase (190, 331); gene cloned and sequenced (27, 190, 217)
CDC9	DNA ligase	SJ	Synthesizes DNA at 36°C but remains partially sensitive to hydroxyurea (140); structural gene for DNA ligase (15, 16, 122, 182); heterogeneous with respect to MBC sensitivity after incubation at 36°C (406); increased rate of mitotic recombination at maximum permissive temp (122, 142)
CDC13	?	DS (?) or ND	Synthesizes DNA at 36°C and becomes insensitive to hydroxyurea (140); independent of MBC-sensitive step (406); increased rates of chromosome loss and mitotic recombination at maximum permissive temp (142)
CDC14	?	DS (?) or ND	Synthesizes DNA at 36°C and becomes insensitive to hydroxyurea (140); execution of MBC-sensitive step required for execution of *CDC14* step (406); increased rates of chromosome loss and mitotic recombination at maximum permissive temp (142)
CDC15	?	DS (?) or ND	Synthesizes DNA at 36°C and becomes insensitive to hydroxyurea (140); heterogeneous with respect to temp sensitivity after incubation with MBC (406); increased rates of chromosome loss and mitotic recombination at maximum permissive temp (142)
CDC16	?	DS (?) or ND	Synthesizes DNA at 36°C and becomes insensitive to hydroxyurea (139, 140); independent of MBC-sensitive step (406); independent of *CDC45* step (258); some alleles temp sensitive for DNA synthesis in permeabilized cells (218); increased rate of chromosome loss at maximum permissive temp (142)
CDC17	Catalytic subunit of DNA polymerase I	DS	Synthesizes DNA at 36°C (139); function prerequisite for execution of MBC-sensitive step (406); increased rates of chromosome loss and mitotic recombination at maximum permissive temp (142); increased telomere length at maximum permissive temp (63); structural gene for catalytic subunit of DNA polymerase I (Carson, Ph.D. thesis); gene cloned and sequenced (178, 234, 301)
CDC20	?	ND	Synthesizes DNA at 36°C (139), independent of MBC-sensitive step (406); increased rate of chromosome loss at maximum permissive temp (142)
CDC21	Thymidylate synthase	PS	Ceases DNA synthesis quickly after temp shift (139); remains sensitive to hydroxyurea (140); heterogeneous with respect to MBC sensitivity after incubation at 36°C (406); structural gene for thymidylate synthase (28, 121); gene cloned and sequenced (372)
CDC23	?	ND	Synthesizes DNA at 36°C (139); becomes insensitive to hydroxyurea (140); execution of MBC-sensitive step required for execution of *CDC23* step (406); no increase in rates of chromosome loss and mitotic recombination (142)
CDC40	?	iDS or DS	Synthesizes DNA at 36°C but remains sensitive to hydroxyurea (193); gene cloned (192)
CDC44	?	ND	Cold-sensitive mutant; synthesizes DNA at 17°C and becomes insensitive to hydroxyurea (259); dependent pathway: *CDC16* → *CDC44* → *CDC14* (258)
CDC45	?	ND	Cold-sensitive mutant; synthesizes DNA at 17°C and becomes insensitive to hydroxyurea; independent of *CDC16* (258)
CDC46	?	ND (?)	Temp sensitive; extragenic suppressor of *cdc45*; no DNA synthesis measurements (259)
CDC47	?	ND (?)	Temp sensitive; extragenic suppressor of *cdc45*; no DNA synthesis measurements (259)
CDC48	?	ND (?)	Temp-sensitive and cold-sensitive alleles; no DNA synthesis measurements (259)
DBF1[c]	?	DS or PS	Ceases DNA synthesis immediately after temp shift; no increase in spontaneous mutation frequency (183)

Continued on following page

TABLE 3—*Continued*

Gene	Product	Function[a]	Remarks and reference(s)
DBF2[c]	?	DS or PS	Ceases DNA synthesis immediately after temp shift; no increase in spontaneous mutation frequency (183)
DBF3[c]	?	iDS or DS	Reduced rate of DNA synthesis after temp shift; no increase in spontaneous mutation frequency (183); in synchronized cells small burst of DNA synthesis at time of initiation of replication (184)

[a] iDS, Initiation of DNA synthesis; DS, DNA synthesis; PS, precursor supply; SJ, strand joining; ND, nuclear division.
[b] MBC, Methyl-(benzimidazole-2-yl)carbamate.
[c] The *DBF* genes were identified as cell cycle mutants that arrest as large budded cells (dumbbell formers). They are not allelic to *cdc1* to *cdc31* mutations (122).

temperature sensitive (218). Based on the observations that replication intermediates were not found in DNA isolated from cells incubated at the nonpermissive temperature and that a random two-thirds of the genome replicated in any given cell, it was suggested that the *CDC2* gene product functions in the initiation of DNA replication. At the nonpermissive temperature, replication would initiate at only a small fraction of origins, and the DNA that fails to replicate would represent chromosomes which failed to initiate replication. This is consistent with the reduction in DNA synthesis in permeabilized cells because fewer replication forks would be active. Further characterization of *cdc6* and *cdc40* mutants may lead to identification of other replication defects.

In both bacteriophage T4 and *E. coli*, mutations in genes required for DNA replication often increase recombination, presumably, in part, because the DNA contains lesions that are recombinogenic (51, 210). In *S. cerevisiae*, mutations in genes known to be required for DNA replication cause increased rates of mitotic recombination, chromosome loss, or both. *cdc9* (DNA ligase) mutants exhibit elevated rats of mitotic recombination (122), and *cdc8* (thymidylate kinase) and *cdc17* (DNA polymerase I) mutants show both phenotypes (142; M. J. Carson, Ph.D. thesis, University of Washington, Seattle, 1987). Therefore, other mutants which display similar chromosome loss and mitotic recombination phenotypes are important candidates for the identification of components of the DNA replication machinery. When this screen was applied to cell cycle mutants blocked in S phase or nuclear division, strains carrying mutations in *CDC13*, *CDC14*, *CDC15*, *CDC16*, and *CDC20* as well as the mutants defective in precursor synthesis and those defective in the hydroxyurea-sensitive step showed one or both of these phenotypes.

Thus, more than half of the mutations defective in some aspect of S phase or nuclear division affect DNA replication. The remaining genes on this pathway (Table 3) may play roles in nuclear division unrelated to DNA replication. However, it is also possible that some may have a role in replication that has not yet been revealed by the screens applied.

Regulation of expression. Experiments in which the wild-type copy of a *CDC* gene is lost either by recombination or plasmid segregation demonstrate that all *CDC* gene products except the *CDC4* product are present in amounts sufficient to support at least several cell divisions (332, B. Byers and L. Sowder, J. Cell Biol. **87**:6, 1980). In the case of the gene products required for transit through G1, this is not surprising since they are probably regulated posttranslationally. For the two genes required for transit through G1 that have been examined (*CDC7* and *CDC36*), the rate of transcription of the gene does not vary during the cell cycle (295, 332).

In contrast, genes whose products are directly required for DNA replication, including *CDC8* (thymidylate kinase), *CDC9* (DNA ligase), *CDC21* or *TMP1* (thymidylate synthase), *CDC17* or *POL1* (the large subunit of DNA polymerase I), and *PRI1* (required for DNA polymerase I-associated DNA primase activity), are transcribed periodically in the cell cycle in late G1 and early S (185, 252, 295, 355, 395). In addition, the activity of ribonucleotide reductase, which is necessary for deoxynucleotide synthesis, peaks at the same time (233). Thus, although these gene products are not present in limiting amounts, it seems likely that optimal levels of DNA synthesis require additional gene product. The transcription of the DNA replication genes occurs somewhat earlier than the periodic transcription of histone genes, and the transcription of the DNA replication genes occurs in *cdc4* strains at the nonpermissive temperature while histone gene transcription does not (185, 395). Therefore, the DNA replication genes may be coordinately controlled, while the histone genes must be regulated separately from the DNA replication genes. Transcription of *CDC8*, *CDC9*, and *RNR2* (the gene that encodes the small subunit of ribonucleotide reductase) is also induced after DNA damage (104, 165, 295), a time when these gene products are expected to be required. There are conflicting reports about the transcription of *POL1* after DNA damage (104, 185). The difference may result from the use of different agents to induce damage in the two studies.

In contrast to *CDC8* and *CDC21*, two other genes encoding enzymes that can function in deoxythymidine triphosphate synthesis, *DCD1* (deoxycytidine monophosphate deaminase) and *DUT1* (deoxyuridine triphosphate pyrophosphatase), may not be cell cycle regulated (252, 253). In these studies the fluctuation in transcript level of the *CDC21* gene was larger than that of either *DCD1* or *DUT1*. Without quantative analysis of hybridization levels, however, it is not possible to eliminate a two- or threefold fluctuation in transcription during the cell cycle.

Biochemistry and Reverse Genetics

Although it seems certain that the collection of cell cycle mutants and additional collections of conditional mutants that have been screened directly for defects in DNA synthesis (97, 181) contain mutants defective in replication proteins not yet identified, there are several reasons to believe that other approaches to the identification of replication genes are necessary. First, some replication mutants may arrest with a nonuniform terminal phenotype and would not be included in collections of cell cycle mutants. This is clearly the case for topoisomerase II mutations (see discussion below). Second, temperature-sensitive mutations may not occur readily in some genes. The distribution of mutations among the cell cycle genes demonstrates that some genes are more susceptible than others to the occurrence of tempera-

ture-sensitive mutations (141). Moreover, genes identified by cold-sensitive cell cycle mutations are largely different from those identified by temperature-sensitive mutations (259). Finally, if two or more genes encode the same product or functionally interchangeable products, then traditional mutant isolation schemes are unlikely to work. Below are summarized biochemical and reverse genetic approaches to replication proteins.

DNA polymerase and DNA primase. *S. cerevisiae* contains four DNA polymerases. Two nuclear enzymes, DNA polymerases I and II and a mitochondrial DNA polymerase, were defined by early fractionation studies (73, 169, 401–403). A third nuclear enzyme, DNA polymerase III, has recently been described (20, 55). DNA polymerase I of *S. cerevisiae* corresponds to DNA polymerase α of higher cells, and polymerase III may be a polymerase δ analog. DNA polymerase I is the most abundant DNA polymerase when extracts are fractionated on diethylaminoethyl cellulose columns and has been studied more extensively than the others. Proteolysis during purification has made it difficult to analyze the structure of the enzyme, but it is now known that enzyme purified by conventional methods (8, 302, 340) and by immunoaffinity chromatography (9, 234, 235, 292, 303) contains four subunits of 180, 86, 58, and 48 kilodaltons (kDa). DNA polymerase activity is associated with the 180-kDa subunit, which copurifies with the 86-kDa protein of unknown function. Both of these polypeptides are often partially degraded. Catalytically active polymerase is found in a series of bands from 140 to 180 kDa, the largest of which is slightly larger than the 166-kDa species predicted from the DNA sequence (301). The 86-kDa polypeptide is often present as a single band of 74 kDa that is probably a degradation product. DNA primase activity is associated with the 58- and 48-kDa subunits and can be dissociated from the larger subunits without loss of primase activity.

DNA polymerase I was first implicated as a replicative polymerase by the isolation of aphidocolin-resistant mutants of *S. cerevisiae* (358). In these mutants, DNA polymerase I is about 20-fold less sensitive to aphidicolin than the wild-type enzyme, while the sensitivity of DNA polymerase II is unaffected. More direct evidence that DNA polymerase I is an essential protein came from studies with the cloned gene, which was identified by antibody screening of an expression library (178, 234). Disruptions of the coding region are lethal in a haploid (178, 305), making it possible to screen for temperature-sensitive alleles of the gene generated by in vitro mutagenesis. Eight temperature-sensitive *pol1* alleles have been studied (54, 236). All of the mutant alleles cause arrest of cell division at the nonpermissive temperature with the phenotype expected of DNA replication mutants, a uniform population of cells with large buds and single nuclei. All of the mutants show significant residual DNA synthesis after shift to the high temperature, with the most severely affected strain showing 25% of wild-type incorporation. Whether this incorporation reflects the activity of other DNA polymerases or results from leakiness of the *pol1* mutations is not clear. The *pol1-1* mutant of Lucchini et al. continues DNA synthesis at the wild-type rate until the beginning of the next S phase, when it shuts off synthesis, suggesting that the mutation results in a polymerase that cannot fold correctly at the nonpermissive temperature but, once assembled, is stable to high temperature (236, 301). Analysis of partially purified *pol1-1* mutant enzyme showed that the polymerase-primase complex is less stable than wild type and that an epitope in the wild-type catalytic subunit recognized by a monoclonal antibody is not present. The *cdc17* mutations, which are also in the *POL1* gene (Carson, Ph.D. thesis), result in a phenotype similar to the *pol1-1* mutant studied by Lucchini et al. and the less severely affected mutants of Budd and Campbell (54). *CDC17* had not been considered a likely candidate for the *POL1* gene because of the extensive residual DNA synthesis in *cdc17* strains and the fact that the requirement for the *CDC17* gene product was not sequenced with respect to the hydroxyurea-sensitive step (140).

The DNA primase activity of the DNA polymerase I-primase complex has been studied extensively (9, 339, 340). The enzyme catalyzes the synthesis of RNA oligomers of 8 to 12 nucleotides. In the absence of deoxynucleotide substrates for DNA polymerase, DNA primase makes longer RNA oligomers that are multimers of the 8- to 12-nucleotide modal length. In the presence of DNA substrates, primer synthesis is tightly coupled to DNA chain extension. A monoclonal antibody that specifically recognizes the smaller protein (48 kDa) associated with primase activity and that inhibits primase activity in vitro has recently been used to clone the gene encoding this subunit (*PRI1*) from an expression library (235). The gene is essential as demonstrated by gene disruptions. The *PRI1* gene has been sequenced, and its predicted protein shows no significant homology to procaryotic DNA primases (304).

There are two reports of a 65-kDa protein with DNA priming activity (176, 400). The relationship of the polymerase-associated primase to the 65-kDa protein remains to be firmly established, although they appear to be different.

DNA polymerase II is less abundant than DNA polymerase I, comprising 10 to 30% of total polymerase activity. It is distinguished from the more abundant enzyme by having 3′-5′-exonuclease activity. Its role in DNA replication is not known.

DNA polymerase III has recently been identified in extracts from protease-deficient strains prepared in the presence of protease inhibitors (20, 55). It has an associated 3′-5′-exonuclease activity and no associated DNA primase activity. The exonuclease is capable of "proofreading," removing single nucleotide mismatches at the 3′ end of a primer that is base paired to a template strand. Purified DNA polymerase III contains several polypeptides including one of 140 kDa and several in the 53- to 62-kDa range. Surprisingly, DNA polymerase I activity is largely absent from these extracts, just as DNA polymerase III is largely absent from extracts that contain large amounts of DNA polymerase I. However, the enzymes are, in fact, different. DNA polymerase III is about 8-fold more sensitive to aphidicolin and 200-fold less sensitive to N^2-(*p-n*-butylphenyl)-2′-deoxyguanosine 5′-triphosphate than DNA polymerase I. In addition, antibodies that inhibit DNA polymerase I have no effect on DNA polymerase III and vice versa. A possible explanation for the failure to recover DNA polymerase I activity in the extracts from protease-deficient strains is that unproteolyzed DNA polymerase I is not active on the activated calf thymus DNA template used for assay. Consistent with this explanation is the observation that DNA polymerase I-associated DNA primase is recovered in the same amount and in the same fractions from the high-pressure liquid chromatography column whether or not the polymerase activity can be detected.

Yeast DNA polymerase III is similar to mammalian DNA polymerase δ in the size of its high-molecular-weight subunit, its 3′-5′-exonuclease activity, and its pattern of inhibitor sensitivities. Its role in DNA replication is not yet known. Recent results implicate DNA polymerase δ as well

as DNA polymerase α as essential for SV40 replication in vitro. Evidence for the δ polymerase requirement is indirect: a protein, proliferating cell nuclear antigen (PCNA), that functions as an auxiliary factor for the δ polymerase is required in the in vitro DNA replication system (309). Further studies of DNA polymerase III and the isolation and mutagenesis of the yeast *POL3* gene will provide insight into the role of DNA polymerase III in yeast DNA replication.

Topoisomerases. Temperature-sensitive mutations in the genes encoding both topoisomerase I (*TOP1*) and topoisomerase II (*TOP2*) of *S. cerevisiae* have been obtained either by screening heavily mutagenized strains for defects in enzyme activity (94, 376) or by in vitro mutagenesis of the *TOP2* gene (155). Strains carrying mutations in *TOP1*, including null mutations, show no obvious growth defects (129, 375, 376). Therefore, topoisomerase I is not essential. *TOP2* is an essential gene (94, 117, 155), and topoisomerase II is absolutely required at the time of mitosis (94, 128, 146). The requirement for topoisomerase II appears to be for disengaging sister chromatids during chromosome segregation. When temperature-sensitive *top2* mutants are incubated at the restrictive temperature, they become inviable at the time of mitosis; however, inviability at the restrictive temperature is prevented by nocodozole, a microtubule polymerization inhibitor that prevents mitotic spindle formation (155). Moreover, *top2* mutations result in large increases in chromosome nondisjunction and some increase in chromosome breakage at the nonpermissive temperature (C. Holm, T. Stearns, and D. Botstein, Mol. Cell. Biol., in press). In contrast to the single mutants, *top1 top2* double mutants grow poorly at the permissive temperature and are defective in DNA replication and transcription of ribosomal RNA as well as mitosis at the nonpermissive temperature (48). These results suggest that, except for chromosome segregation, the topoisomerases can substitute for each other. The essential function that can be provided by either topoisomerase is presumably to provide a swivel to relieve the torsional stress that results from unwinding the DNA helix during DNA replication and transcription. Although the two enzymes use different strand passing mechanisms, either enzyme is capable of relieving the torsional stress induced in the 2μm plasmid by cooling cells to 0°C (324). *Schizosaccharomyces pombe* mutants defective in topoisomerases I and II have phenotypes similar to those of the corresponding *S. cerevisiae* mutants (382, 383).

The topoisomerase genes provide an excellent example of the importance of using several approaches for mutant isolation. Although the *TOP2* gene is essential and required at only one time during the cell cycle, temperature-sensitive *top2* mutants do not arrest with a uniform phenotype. At the nonpermissive temperature, unbudded cells as well as cells with large buds accumulate. The budded cells are arrested in nuclear division and the unbudded cells have completed an aberrant division (155). Therefore, *top2* mutants would not be recovered in a screen for cell cycle mutants. In addition, since *top1* mutants have a growth phenotype only in the presence of a *top2* mutation, the biochemical screening and reverse genetic approaches to mutant isolation were the most efficient approaches. The *MAK1* gene, which is required for the maintenance of the double-stranded RNA killer plasmid, is *TOP1* (376). However, it was far from obvious that a topoisomerase I deficiency would cause this particular phenotype. The mutants have been important tools for understanding the in vivo function of these enzymes.

Single-stranded DNA-binding proteins. Single-stranded DNA-binding proteins (or single-stranded nucleic acid-binding proteins, SSBs) apparently lack enzyme activity and bind more strongly to single-stranded DNA in a non-sequence-specific manner than to double-stranded DNA. The requirement for SSBs in procaryotic DNA replication is well documented (reviewed in reference 75), and it has therefore been of interest to identify and characterize yeast SSBs.

Four groups have reported the purification of SSBs that stimulate the activity of yeast DNA polymerase I (7, 74, 187, 219). These proteins are similar in apparent molecular weight (estimates vary from 37,000 to 45,000), but it is not known whether they are related. The SSB1 protein purified by LaBonne and Dumas (219) and the SSB1 protein purified by Jong et al. (187) are similar in amino acid composition but differ in the extent and mode of stimulation of DNA polymerase I. Using antibodies specific for their SSB1, Jong and Campbell cloned the *SSB1* gene (188). The gene appears not to be essential; strains carrying a disruption of the gene are viable, grow normally, and apparently lack protein recognized by antibodies to SSB1 protein. However, the gene disruption removes only 27 carboxy-terminal amino acids from the SSB1 protein and the authors think the requirement for *SSB1* should be reexamined (189). SSB1 protein has been localized to the nucleolus by immunofluorescence microscopy, and its predicted amino acid sequence shows homology with several heterogeneous nuclear RNA-binding proteins (189). Thus, SSB1 may function in RNA rather than DNA metabolism.

Four other yeast SSB proteins have been identified and two of them, SSB2 and SSBm, have been purified (187). SSB2 binds DNA more tightly than SSB1, and SSBm appears to be a mitochondrial protein. None of these SSBs has yet been studied further.

Helicases. Helicases are DNA-dependent adenosine triphosphatases (ATPases) that separate strands of duplex DNA. The *RAD3* gene has been shown to encode a DNA helicase (362). The *RAD3* gene product is required for excision repair of damaged DNA (reviewed in reference 116). Gene disruptions and the recent isolation of a *rad3* mutant that is temperature sensitive for growth demonstrate that this gene has an essential function (151, 271, 272). Whether the helicase activity is itself essential is not yet known. ATPase III, a second ATPase with helicase activity, has been purified from *S. cerevisiae* (359). Surprisingly, although extracts of *rad3* strains are deficient in ATPase III, the *RAD3* gene product and ATPase III have different molecular weights, and antibodies that precipitate the *RAD3* protein do not precipitate ATPase III. These observations suggest that the *RAD3* gene product is not ATPase III.

In Vitro DNA Replication Systems

Faithful in vitro DNA replication systems have been important for purifying proteins required for DNA replication as well as for understanding the mechanisms of action of a number of replication enzymes. The first yeast in vitro systems were cells permeabilized with nonionic detergent that were capable of elongating replication forks initiated in vivo when incubated in the presence of added deoxyribonucleotide triphosphates (12, 146, 216). Surprisingly, DNA synthesis is temperature sensitive in permeabilized *cdc8* strains which are defective in thymidylate kinase (146, 190, 216). This system was used in a complementation assay for the partial purification of a protein that suppresses the *cdc8* defect (216). While purified thymidylate kinase has been reported to complement the *cdc8* defect in permeabilized

cells, whether it was among the proteins purified by the complementation assay has not been reported (190). Whether thymidylate kinase is required for deoxythymidine triphosphate regeneration in the system or whether it serves an essential role in the replication complex is not yet known (see above).

The permeabilized cell system has also been used to screen for mutants defective in DNA synthesis (217). Twenty mutants were identified among a collection of 400 strains carrying temperature-sensitive lethal mutations. Seven of the mutations were found to be allelic to previously identified mutations in the *CDC2*, *CDC8*, and *CDC16* genes (Table 3). The remaining 13 mutants have not been studied further.

Cell-free extracts capable of in vitro DNA synthesis have been described by several groups (65, 173, 209, 333). DNA synthesis in all of these extracts is temperature sensitive when the extracts are prepared from *cdc8* strains. However, the protein purified by Arendes et al. that complemented the temperature sensitivity of a *cdc8* extract was reported to be a single-stranded DNA-binding protein with a molecular weight of 37,000 rather than the *CDC8* gene product, thymidylate kinase, which has a molecular weight of 25,000 (7). The complementing activity prepared from *cdc8* strains was temperature sensitive. How these observations relate to the in vivo function of the *CDC8* gene product is unclear.

In at least two of the extracts, the DNA replication activity is associated with a high-molecular-weight (>1,000,000) complex that has been reported to contain DNA polymerase I, DNA ligase, DNA primase, topoisomerase II, and ribonuclease H activity (175, 360).

While all of the extracts have been reported to use preferentially an *ARS*-containing DNA fragment as template, none of the systems is dependent on an *ARS*-containing template. The positions of replication bubbles presumably initiated in vitro have been mapped by electron microscopy. In products from the Sugino extract, bubbles corresponding to the 2μm plasmid *ARS* and to a specific region of the pMB9 vector accounted for about 80% of the replication structures (209). Celniker and Campbell mapped the position of replication bubbles to the *ARS* of the *ARS1* plasmid template (65). In addition, Jazwinski et al. (174, 177) reported that their replication extracts contained a high-molecular-weight complex that could be seen by electron microscopy to bind to two regions of 2μm DNA that had been reported to act as origins in vivo (278) and to a single region coincident with the *ARS* of an *ARS1*-containing plasmid. These binding regions were coincident with the positions of replication bubbles found in samples incubated under replication conditions.

However, for one of these extracts (333), it was later reported that DNA synthesis in the extract was dependent upon oligonucleotide primers present in CsCl-purified plasmid templates prepared from *E. coli* (191). While other extracts have been reported to work as efficiently on alkali-treated templates as on untreated templates, and thus may be initiating DNA replication in vitro (65, 175), these systems are not efficient. In the cases for which quantitative data have been presented, <5% of DNA molecules examined had replication structures (174, 175, 177, 209).

Although these systems are promising, none is yet in general use by other laboratories in the field. Clearly, the further development and improvement of yeast in vitro replication systems will be important for progress in understanding the enzymatic properties of replication proteins and their contributions to the process of DNA replication.

CENTROMERES

Centromeres are the regions of chromosomes to which spindle fibers attach to effect segregation of chromosomes at cell division. Genetically, centromeres are recognized by their ability to direct the mitotic segregation and first meiotic division segregation of genes adjacent to them. Cytologically, centromeres are visualized as a primary constriction in condensed chromosomes. Particularly in cytological studies, the centromeric region of chromosomes has been referred to as the kinetochore. Ultrastructural analysis of kinetochores of higher cells has shown them to have a trilaminar structure composed of protein and nucleic acid to which bundles of microtubules attach (318). In many lower eucaryotes the structural differentiation of the centromeric region is not apparent (reviewed in reference 215). Ultrastructural studies of yeast chromosomes have revealed that a single microtubule binds to yeast chromatin in a structurally undifferentiated region (294).

Cloning and Biological Properties

Cloning and segregational analysis. The first yeast centromere was identified in a DNA fragment from chromosome III that contains the *CDC10* gene which is tightly linked to *CEN3* (79). Evidence that the fragment contained *CEN3* was provided by both its location adjacent to centromere-linked genes on chromosome III and its biological properties. The *CEN*-containing fragment acted in *cis* to stabilize mitotically an *ARS*-containing plasmid and also directed the plasmid to segregate during the first meiotic division. Thus, in a majority of tetrads, the plasmid segregated into two sister spores.

Centromeres are essential for chromosome stability. Deletion of *CEN3* (80) or its replacement by a conditional centromere (152) results in rapid loss of the chromosome. Replacement of *CEN3* with *CEN11* or inversion of *CEN3* has no effect on mitotic or meiotic chromosome stability (80). These results suggest that centromeres are not chromosome specific and that they function normally in either orientation. The centromere replacement experiments have been done only in euploid strains. Since there is evidence that *S. cerevisiae* has a distributive disjunction system that segregates unpaired chromosomes in meiosis (89, 241), it would also be interesting to examine the centromere replacements in a trisomic strain in which the third copy of chromosome III carried *CEN11* instead of *CEN3*. This would test the ability of the chromosome carrying *CEN11* to pair and segregate in direct competition with the chromosomes carrying *CEN3*.

The growth of transformants carrying *ARS*-containing plasmids on nonselective medium results in rapid loss of the plasmid from the culture, indicating that these plasmids are mitotically unstable (see above). The ability of a centromere to stabilize the inheritance of a plasmid provides a direct selection for centromeric DNA (151, 160, 239). After transformation with a yeast library constructed in an *ARS*-containing vector and prolonged growth of the transformants under nonselective conditions, a large fraction of plasmid-containing cells carry plasmids with centromeres. Twelve of the 16 *S. cerevisiae* centromeres have now been cloned by either selecting for tightly linked genes or selecting DNA fragments capable of stabilizing *ARS* plasmids (79, 113, 150, 160, 162, 239, 255, 274, 290, 350, 351, 377).

With one exception, the properties of the cloned centromeres that have been examined are similar to those of *CEN3*. All of the cloned centromeres have the ability to

direct mitotic segregation of plasmids. Two centromeres, *CEN3* and *CEN4*, have been assayed in vectors that allow a distinction between 1:0 (simple loss) and 2:0 (nondisjunction) mitotic loss events and both show similar rates of nondisjunction (149, 305). Seven centromeres have been assayed for their abilities to direct meiotic segregation of plasmids. All plasmids tested segregate predominantly 2:0 in meiosis with varying fractions of 4:0 and 0:4 segregations. The 3:1 and 1:3 tetrad classes are rare (79, 159, 113, 114, 239, 274, 351, 377). In the case that was examined directly, the 4:0 and 0:4 classes quantitatively reflected the fraction of cells in the mitotic culture that was sporulated that either contained two copies of the plasmid or had lost the plasmid (212). All but one of the *CEN*-containing plasmids examined segregated predominantly into sister spores at meiosis I, as expected for first-division segregation. Thus, the meiotic segregation of these plasmids mimics chromosomal segregation. However, a plasmid containing *CEN15* showed about 65% second-division segregation, consistent with premature disjunction at meiosis I (377). It will be important to confirm this unexpected result, which suggests that sequences important for meiotic segregation of chromosome XV lie outside the sequences required for mitotic stability.

The close association of several centromere-linked genes with their respective centromeres (79, 113, 255, 290, 350, 351) and the mapping of transcripts around four centromeres (243, 350, 351, 410) have revealed that, in contrast to higher cells, the regions flanking *S. cerevisiae* centromeres are transcriptionally active and are free of highly repetitive satellite DNA.

Dicentric plasmids and chromosomes. The classic work of McClintock showed that, when dicentric chromosomes are formed in *Zea mays* as a result of fusion of broken chromosomes, the dicentrics are unstable, often being broken again in subsequent mitotic divisions when the two centromeres attach to opposite poles of the spindle (249, 250). They continue in this "breakage-fusion-bridge" cycle until the broken ends are ultimately healed by the acquisition of new telomeres. Dicentric plasmids and chromosomes are also unstable in *S. cerevisiae*, and the dicentrics are ultimately stabilized by rearrangements that delete one of the centromeres (134, 135, 213, 241, 284, 365; A. Hill and K. Bloom, personal communication). However, in contrast to dicentric chromosomes in *Z. mays*, both dicentric plasmids (213, 241) and dicentric chromosomes (135) can be propagated for many generations before they are broken.

Dicentric plasmids are nevertheless mitotically unstable. Less than 10% of cells grown under selective conditions have plasmid, and the plasmid-containing cells each have about seven copies of the plasmid (213). The mitotic instability of dicentric plasmids is most easily explained by their frequent nondisjunction. Plasmid nondisjunctions might be expected to occur when the centromeres of a dicentric plasmid are attached to opposite poles of the spindle. In this situation, the tension generated by the plasmid being pulled to opposite poles might not be limited to breakage of the plasmid DNA, but might alternatively break one of the spindle microtubules attached to the plasmid. If a microtubule breaks at random, then a nondisjunction would result half of the time.

The rate at which dicentric plasmids with two copies of the same centromere rearrange to yield monocentrics depends upon the relative orientation of the centromeres (213, 241). When the centromeres are inverted with respect to each other, rearrangements are produced at a rate of 10^{-3} per cell division. The structure of the rearranged plasmids is consistent with the occurrence of a pair of double-strand breaks that separate the centromeres followed by ligation of the broken ends to regenerate a circular plasmid. When the centromeres are in the direct orientation, monocentric rearrangements result from a homologous recombination between sequences flanking the centromeres, and they occur too rapidly for an accurate measurement of rate. When centromeres are in the inverted orientation, monocentric plasmids accumulate more rapidly in the culture than expected from the rate of rearrangement. This apparent discrepancy between the low rate of plasmid rearrangement and the fast accumulation of monocentric plasmids can be explained by the observation that cells harboring only monocentric plasmids grow much faster than cells carrying dicentric plasmids.

The longer doubling time of cells harboring dicentric plasmids could result from delays in the completion of mitosis because a plasmid whose centromeres are attached to both poles of the spindle cannot segregate until the bipolar attachment is resolved by breakage of the plasmid or the spindle fibers. Alternatively, the extra centromeres present in cells harboring several copies of the plasmid could impede the segregation of chromosomes by competing for components of the segregation apparatus present in limiting amounts. This second hypothesis is analogous to that proposed by Futcher and Carbon to explain the toxic effects of maintaining multiple monocentric plasmids (119). Support for the first hypothesis is provided by the recent demonstration by Hill and Bloom (personal communication) that activation of an integrated conditional centromere to make chromosome III dicentric results in the lengthening of the cell cycle and accumulation of cells in G2. The importance of the second hypothesis is unclear. When the copy number of a plasmid containing a conditional centromere was increased by inactivating the centromere, and the copy number of the plasmid was monitored as a function of time after the centromere was reactivated, the *CEN* plasmid stabilized at four to five copies per cell (152). Therefore, it appears that yeast cells can tolerate a modest increase in centromere number. However, it is unclear why Futcher and Carbon failed to see an effect on the growth rate of the cells after activating the conditional centromere in a conditionally dicentric plasmid. Perhaps further analysis of recently reported mutants that tolerate centromere plasmids at high copy number (381) will help to resolve this issue.

The instability of dicentric plasmids probably explains why 2μm plasmids containing cloned centromeres are unstable (380). This plasmid normally replicates once per cell cycle (417), but it is capable of amplifying when the copy number is low. Amplification occurs when an intramolecular recombination within a replicating molecule produces a double-rolling-circle intermediate that replicates to yield tandem multimers of the plasmid (117, 316, 389). If the plasmid carries an integrated centromere, then the amplification products would be multicentric and might be expected to produce the kinds of deletion derivatives found.

The instability of dicentric plasmids is tempered significantly by moving the centromeres close together (213). An increase in stability is first seen in constructs in which the centromeres are within approximately 1 kb of each other. Plasmids in which the centromeres are separated by only 96 bp approach the stability of monocentric plasmids. These results suggest that either one centromere may inhibit the function of a second in close proximity or the function of the two centromeres can be coordinated. These observations provide a potential explanation for the mechanism by which centromeres with multiple microtubule binding sites could

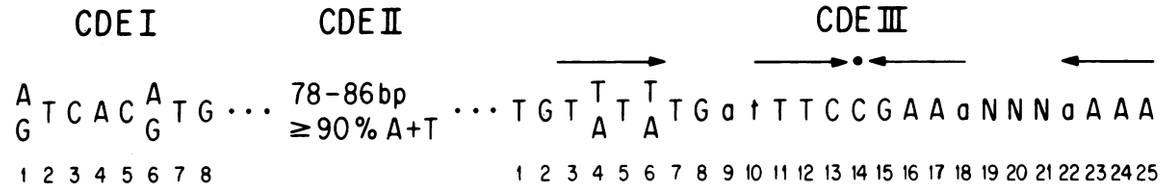

FIG. 4. Centromere DNA sequences. Centromere DNA elements I, II, and III were defined by Fitzgerald-Hayes et al. (114) on the basis of comparisons between the sequences of *CEN3* and *CEN11* and modified by Hieter et al. (150) on the basis of comparisons of 10 centromere sequences. Nucleotides that are capitalized are conserved in at least 9 of 12 sequences. Nucleotides in lowercase letters are present in at least half of the centromere sequences. Positions with a variable nucleotide are represented by an N.

have evolved from centromeres with a single microtubule binding site such as those in *S. cerevisiae*.

Studies of the resolution of dicentric chromosomes in *S. cerevisiae* suggest that the mechanisms are similar to those that resolve dicentric plasmids. Chromosome breakage followed by healing of the broken ends (134) as well as resolution by intrachromosomal recombination between repeated sequences, either Ty elements (364) or sequences flanking the centromere (Hill and Bloom, personal communication), have been observed.

***CEN* effects on mitotic and meiotic recombination.** In *Drosophila melanogaster*, comparisons of physical and genetic chromosome maps (95) and genetic analysis of chromosomal rearrangements that alter the distance between the centromere and other regions of the chromosome (23, 408) provide strong evidence that the centromere suppresses meiotic recombination in its vicinity.

In *S. cerevisiae*, three lines of evidence are consistent with a centromeric suppression of meiotic recombination, but none of the evidence is unequivocal. First, on chromosome VII, the frequency of mitotic crossovers between *CEN7* and a closely linked marker, *LEU1*, is sixfold higher than the frequency of meiotic crossovers (240). These observations could result from either meiotic suppression or mitotic stimulation of recombination. The recent description of a mitotic recombination hot spot adjacent to *CEN14* is consistent with the latter interpretation (275).

Second, the frequency of meiotic exchange in some, but not all, centromeric regions for which physical maps are available is below the average genomic frequency (79, 255, 290, 351; D. Kaback, personal communication). However, other chromosomal intervals, distant from a centromere, also have meiotic exchange frequencies well below the genomic average (222, 366).

Third, when Lambie and Roeder moved *CEN3* to a new position on the left arm of chromosome III, they found that the frequency of meiotic exchange increased in an interval near the original position of the centromere and decreased in an interval adjacent to its new position (220). These results suggest that the centromere either has a suppressive effect on meiotic recombination in its vicinity or is important in determining the overall structure of the chromosome, which in turn influences recombination frequencies.

Symington and Petes found that both reciprocal recombination in an interval adjacent to *CEN3* (366) and meiotic gene conversion of *CEN3* (367) occur at rates similar to other genomic sequences. In contrast, Lambie and Roeder have shown that, in a diploid carrying one copy of chromosome III with *CEN3* in its usual position and one copy of chromosome III with *CEN3* moved adjacent to *his4*, the centromere at *his4* acts in *cis* to inhibit gene conversion of adjacent sequences (221). These observations can be resolved in the normal rates of recombination observed by Symington and Petes are, in fact, lower than they would be if *CEN3* were somewhere else. Alternatively, it is possible that the heterologies used by Symington and Petes (366, 367) to monitor exchange induced higher levels of gene conversion, as has been reported by Borts and Haber for another region of chromosome III (38).

Structure-Function Analysis

DNA sequence homology. The DNA sequences of 12 yeast centromeres have now been determined (114, 150, 162, 243, 290). Although homology between centromeres has not been detected by hybridization analysis, centromeres contain conserved sequence elements illustrated in Fig. 4 (114, 150). An 8-bp conserved sequence, centromere DNA element I (CDEI) is separated from a 25-bp conserved sequence, CDEIII, by a 78- to 86-bp region, CDEII, which is >90% A+T but whose exact sequence varies from centromere to centromere. CDEII is characterized by runs of A's and T's six or seven bases in length.

The sequences required for centromere function probably do not extend beyond the 110- to 120-bp region of sequence homology. Because small centromere-containing plasmids are about 3 orders of magnitude less stable than full-length chromosomes (discussed in section on *ARS* assays above), meaningful assessments of full centromere function must be made by replacement of a wild-type centromere on a chromosome (80) or by the creation of large (ca. 150-kb) chromosomal fragments carrying the centromere of interest (144). With these methods, it has been shown that deletion from chromosome III of a 627-bp fragment containing *CEN3* abolishes centromere function (80). The smallest fragment having full centromere function so far reported is a 211-bp fragment containing the elements of homology with 64 bp of DNA flanking CDEI and 28 bp of DNA flanking CDEIII (251). Substitution of a 139-bp fragment with 10 bp flanking CDEI and 4 bp flanking CDEIII results in a 5- to 10-fold increase in the rate of chromosome loss (282). The sequences whose deletion affects the loss rate are likely to be those flanking CDEIII since a 289-bp *CEN3* fragment with only 10 bp of DNA flanking CDEI has full centromere function (59). Similar conclusions regarding *CEN6* have been discussed by Hegemann et al. (144).

Mutational analysis. The effects of deletion, insertion, and point mutations in the regions of homology of three centromeres demonstrate that all three elements contribute to centromere function. CDEIII is essential for mitotic centromere function. Single-base-pair changes within the central region of dyad symmetry (positions 13, 14, and 15, Fig. 4), as well as deletion of several nucleotides on the right and left boundaries of CDEIII completely abolish centromere function in plasmids and chromosomes (123, 143, 144, 251, 282). Chromosome stability is reduced 10^4-fold by these muta-

tions. One- or 2-bp insertions between the center of dyad symmetry and the AAA sequence that forms the right boundary of the dyad also abolish centromere function (144), demonstrating that spacing between the conserved bases is important. Not all conserved bases are equally important, however. Single-base-pair substitutions or 2-bp substitutions at positions 2, 3, 8, 9, 23, 24, and 25 result in rather modest 2- to 10-fold decreases in centromere function.

Although the CDEI sequence is highly conserved, complete deletion of the element (85, 289) and point mutations in the element (144) result in only 5- to 10-fold decreases in mitotic centromere function.

CDEII is also less sensitive than CDEIII to mutational alteration, although deletions, insertions, and substitutions have an effect on centromere function. Deletions that remove half the element and insertions that increase its length by 1.5- to 2-fold decrease chromosome stability by a factor of 10 to 1,000 (59, 85, 123). Centromere function of CDEII deletion mutants can be partially rescued by insertion of foreign DNA (55, 80, 118). A+T-rich DNA is more effective at rescuing function than G+C-rich DNA, suggesting that the high A+T content of this element is important for function.

Several CDEII and CDEIII mutations have been assayed for their abilities to compete with a wild-type centromere in a dicentric plasmid (213). The ability of centromeres carrying CDEIII mutations to compete increased monotonically with their ability to function alone in a chromosome. In contrast, centromeres carrying CDEII mutations that function at almost wild-type levels when substituted in a chromosome were very ineffective competitors of a wild-type centromere in a dicentric plasmid. These results suggest that CDEII and CDEIII may be playing different roles in centromere function.

In addition to mutations within the conserved centromeric sequence elements, centromere function can be abolished by placing a strong promoter adjacent to a centromere (76, 152, 288). In constructs containing a regulated promoter, centromere function is conditional (76, 152).

Several centromere mutations that have modest effects on mitotic chromosome segregation have large effects on meiotic plasmid and chromosome segregation (59, 85; A. Gaudet and M. Fitzgerald-Hayes, Genetics, in press), causing mutant sister chromatids to separate at meiosis I instead of meiosis II. This premature disjunction is seen for both plasmids and chromosomes carrying CDEII mutations, and CDEI deletions cause premature segregation of plasmids but not of chromosomes (59, 85; Gaudet and Fitzgerald-Hayes, in press). In another study, one of the five CDEIII mutations examined resulted in premature segregation of plasmids (283). Thus, CDEII in particular may function in meiosis I to hold sister chromatids together.

Centromere structure in other yeasts. Centromeric DNA has been identified in two other yeasts, *Saccharomyces uvarum* (162) and *Schizosaccharomyces pombe* (78, 111, 270). While *S. uvarum* is sometimes classified as a member of the *S. cerevisiae* species (17), it is not closely related on the basis of DNA homology (162). Two DNA fragments were recovered from a *S.uvarum* library that were capable of mitotically stabilizing a plasmid in *S. cerevisiae*. Both fragments contained a region homologous to the 120-bp centromere of *S. cerevisiae*. Although their function was not assayed in *S. uvarum*, these results suggest that *S. uvarum* centromeres are very similar to those of *S. cerevisiae*.

The fission yeast *Schizosaccharomyces pombe* is not closely related to *S. cerevisiae*. *Schizosaccharomyces pombe* has only three chromosomes. Using cloned centromere-linked genes, two groups have used overlap hybridization to obtain plasmids carrying *Schizosaccharomyces pombe* centromeric DNA (78, 111, 270). *Schizosaccharomyces pombe* centromeres are strikingly different from *S. cerevisiae* centromeres. They contain long (>30-kb) regions of repetitive sequences, making them appear more similar to the centromeric heterochromatin of mammalian cells. It has recently been shown that long centromeric fragments (65 and 105 kb) from *Schizosaccharomyces pombe* chromosomes I and III direct proper meiotic segregation of plasmids in *Schizosaccharomyces pombe* (K. M. Hahnenberger, M. P. Baum, C. M. Polizzi, J. Carbon, and L. Clarke, Proc. Natl. Acad. Sci. USA, in press). It should now be possible to examine the role of the repetitive sequences, if any, in centromere function.

trans-Acting Factors

That *S. cerevisiae* centromeres interact with proteins in vivo has been shown by analysis of centromeric chromatin (34). Centromeric DNA is found in a 220- to 250-bp nuclease-resistant region. This centromere core particle is found in chromosomal and plasmid chromatin and is abolished by both deletions that remove the centromere (35) and point mutations in CDEIII that abolish centromere function (327). Thus, the nuclease-resistant structure is correlated with a biologically active centromere and CDEIII is of crucial importance in the formation or maintenance of the structure.

The centromeric core particle is flanked by a highly ordered array of nucleosome subunits whose phasing is apparently determined by the flanking DNA structure rather than by the centromeric core particle (34, 35).

The core particle can be dissociated by treatment with 0.75 to 1.25 M NaCl, a treatment which also dissociates histones from DNA. The observation that small centromere-containing plasmids are supercoiled to the same extent in vivo as plasmids without centromeres suggests that centromere DNA is wound around proteins in a manner analogous to the winding of DNA around a histone core to form a nucleosome (33).

As in the case of *ARS*-binding proteins, two approaches are being taken to identify and characterize the proteins that interact with centromeric DNA: isolation of *CEN*-binding proteins and isolation of mutations affecting chromosome transmission. Proteins that bind to CDEI (41) and CDEIII (143, 282) have recently been reported. The CDEI-binding protein is rather abundant (at least 500 copies per cell) and binds to the 5'-flanking region of several genes in addition to CDEI. Neither its role in centromeric function nor its relation to proteins that produce the nuclease-resistant centromeric core particle is known. CDEIII-binding protein(s) has been detected by both gel mobility shift assays and exonuclease III blockage assays. The specific DNA-binding activity is inhibited by oligonucleotides containing the CDEIII sequence, and *CEN3* DNA containing a point mutation in CDEIII that abolishes centromere function fails to bind the protein or to compete with wild-type *CEN3* DNA for binding. Thus, the properties of this CDEIII-binding protein suggest that it is biologically significant in centromere function.

trans-acting genes that affect the fidelity of chromosome transmission are expected to encode structural components of the chromosome segregation apparatus as well as proteins involved in aspects of DNA replication and chromosome structure. McGrew and Fitzgerald-Hayes have identified

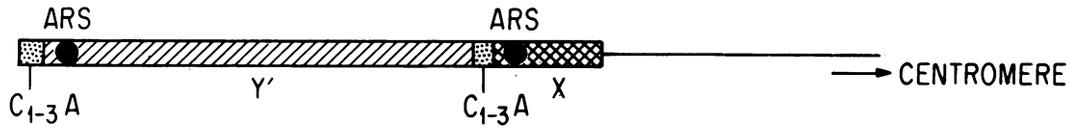

FIG. 5. DNA sequence organization at *S. cerevisiae* telomeres. All yeast telomeres have a terminal tract of several hundred base pairs of $C_{1-3}A$ repeats. In many but not all telomeres, this $C_{1-3}A$ tract is bordered by one or more copies of the 6.7-kb Y′ sequence. Most telomeres have an X sequence. In telomeres with both Y′ and X sequences, these sequences are separated by a tract of $C_{1-3}A$ repeats. Both Y′ and X contain *ARS* elements. Symbols: ▨, $C_{1-3}A$; ▨, Y′; ▩, X; ●, *ARS*.

mutations that fall into four complementation groups and further destabilize a chromosome carrying a weak centromere mutation without significantly affecting wild-type chromosome stability (J. McGrew, Ph.D. thesis, University of Massachusetts, Amherst, 1987). Spencer et al. have used a colony-sectoring phenotype produced by loss of a chromosome fragment marked with *SUP11* to isolate mutants that exhibit increased chromosome loss (347). Six of these mutations are conditional lethal, and they define four complementation groups. Whether any of these mutations are in genes that encode kinetochore proteins remains to be determined. Further analysis of these mutants is likely to increase our understanding of chromosome segregation.

Other Stabilizing Sequences

In addition to centromeres, three DNA sequences capable of increasing the mitotic stability of *ARS*-containing plasmids have been described. A sequence adjacent to *CEN4* that weakly stabilizes plasmids has been described by Mann and Davis (243). This sequence does not detectably compete with a centromere on the plasmid as evidenced by the stability of plasmids carrying both a centromere and this stabilizing sequence.

In contrast, two other sequences, the *STB* locus of the 2μm plasmid and the *HMR E ARS*, that are capable of mitotically stabilizing plasmids do compete with a centromere. Plasmids carrying either of these sequences and a centromere show the same sort of instability as dicentric plasmids (206, 380). The *STB* locus is the *cis*-acting component of the 2μm plasmid partition system whose function also requires the products of the 2μm-encoded *REP1* and *REP2* genes (64, 170, 199). The *STB* locus is capable of stabilizing an *ARS1* plasmid provided the *REP1* and *REP2* gene products are supplied in *trans* (199). The *REP1* gene product is a nuclear protein and appears to be associated with the nuclear matrix (407). Whether the partition system acts to bind plasmids to components that promote their segregation or to free plasmids from sites that would otherwise act to keep all copies of the plasmid with the mother cell has not yet been determined. The segregation function associated with the *HMR E ARS* has been discussed in the section on *ARS* structure-function above.

TELOMERES

Telomeres are the specialized structures at the ends of linear chromosomal DNA molecules. Specialized structures are required because all known DNA polymerases synthesize DNA only in the 5′ to 3′ direction and because no known DNA polymerase can initiate replication without a primer. Accordingly, one strand of the DNA duplex at each end of the molecule could not be replicated completely if it were simply the blunt end of a DNA molecule. In addition, it has been known since the pioneering work of Muller in *Drosophila* spp. (263) and McClintock in corn (249, 250) that the ends of broken chromosomes are very unstable and must be protected from fusion with the ends of other broken chromosomes, recombination with other sequences, or degradation.

Ends of broken DNA molecules are also unstable in yeasts. Dicentric yeast chromosomes undergo the breakage-fusion-bridge cycle described by McClintock (134, 135). Furthermore, when linearized plasmids are used to transform *S. cerevisiae*, two types of transformants are obtained (148, 285). If the DNA ends are homologous to chromosomal sequences, the transformants contain plasmid stably integrated into the homologous chromosomal locus. If the DNA ends have no homology to chromosomal sequences, then transformants containing circular plasmids are recovered. The circular plasmids frequently have deletions of various sizes at the site of the initial break. Since telomeres are stable, they must have a structure or a sequence that distinguishes them from other DNA ends.

Isolation and Structure

The cloning of *S. cerevisiae* telomeres was made possible by the observation that telomeres from *Tetrahymena* or *Oxytrichia* rDNA, when attached to the ends of a linearized *ARS*-containing plasmid, support replication and transmission of the plasmid as a linear molecule in *S. cerevisiae* (87, 306, 370). Yeast telomeres were identified as DNA fragments that could substitute for a *Tetrahymena* telomere on these linear plasmids (370). Analysis of three independently isolated linear plasmids carrying yeast DNA ends suggested that yeast telomeres are all similar in structure. The three yeast fragments had identical restriction maps. Moreover, when one of these fragments was used to probe genomic DNA digests, it appeared that it was repeated about 30 times.

The structure of a typical *S. cerevisiae* telomere is shown in Fig. 5. All chromosomes terminate with a few hundred base pairs of an irregular sequence, one strand of which can be represented as $(C_{1-3}A)_n$ (336, 391). The X and Y′ sequences were initially identified by Chan and Tye as a family of moderately repetitive *ARS* elements (70, 71). Y′ is a highly conserved 6.7-kb element, part of which was shown to be present in cloned telomeres (72). X is a less highly conserved 0.3- to 3.75-kb element. Evidence for $C_{1-3}A$ tracts between X and Y′ is from analysis of clones that were identified by their ability to hybridize a $(GT)_n$ probe and subsequent analysis of X and Y′ clones (391).

The number of Y′ elements varies from zero to four at wild-type telomeres (57, 72, 157, 391, 415). Neither X nor Y′ is essential for normal chromosome function. Murray and

Szostak constructed derivatives of chromosome III lacking both X and Y' that are as stable as the wild-type chromosome (269). In addition, chromosome I lacks both X and Y' in at least two wild-type strains (415).

The sequences necessary for telomere function are the repetitive simple sequence tracts. Telomeres have now been cloned from at least nine protozoa and two slime molds (reviewed in reference 29) as well as the plant *Arabidopsis thaliana* (317). Although the precise sequence varies from organism to organism, they all conform to the general formula proposed by Blackburn (29), $[C_{1-8}(A/T)_{1-4}]_n$. The C-rich strand is oriented 5' to 3' toward the centromere. *S. cerevisiae* adds its own telomeric repeat to linear plasmids carrying *Tetrahymena* (C_4A_2) or *Oxytrichia* (C_4A_4) telomeres (306, 336, 393) but not to linear molecules with other ends (370). In addition, a circular plasmid that carries inverted copies of *Tetrahymena* telomeres is resolved to produce linear plasmids when transformed into yeasts (266, 269, 369).

Telomere Dynamics

Individual telomeres are dynamic structures, varying both from cell to cell in a single strain and from strain to strain. Within a single strain, most of the heterogeneity is from variation in the length of the terminal $C_{1-3}A$ tract. Variation among strains is from heterogeneity in both the length of the terminal tract and the number of Y' sequences.

There is evidence that the length of the $C_{1-3}A$ tract is under genetic control. Genetic crosses between haploid strains having different average terminal tract lengths yielded haploid progeny with a variety of different telomeric lengths, suggesting multigenic control of telomere length (157, 392). Furthermore, mutations in three genes that result in changes in telomere length have been described. Mutations in the *TEL1* and *TEL2* genes, which have no other obvious phenotype, result in short telomeres (237), while strains carrying a temperature-sensitive *cdc17* mutation have longer than normal telomeres when grown at semipermissive temperatures (63). It remains to be determined whether the role of DNA polymerase I (the *CDC17* gene product) in telomere metabolism is direct or indirect. The products of the *TEL* genes have not yet been identified. One possible candidate for a *TEL* gene product is a telomere-binding protein recently described by Berman et al. (25).

While the average length of the terminal tract is under genetic control, there is still substantial heterogeneity in terminal tract length within a genetically homogeneous strain. Two lines of evidence suggest that this heterogeneity is the result of shortening and lengthening activities acting on individual telomeres during each replication cycle. First, the expression of all three telomere length mutations shows a long phenotypic lag, suggesting that telomere length changes only slightly with each replication (73, 237). Second, when individual telomeres were examined in clonal populations of the same strain, telomere length varied from clone to clone, suggesting that telomere length within a clone was determined by the length of the telomere in the cell that gave rise to the clone. Consistent with this hypothesis was the observation that the length heterogeneity of an individual telomere increased with the number of cell divisions following the initial cloning (335). Thus, genetic control appears to establish the equilibrium between shortening and lengthening activities that act on telomeres in each cell cycle.

The strain-to-strain variation in the number and location of Y' elements probably results from frequent recombination between Y' elements. There is evidence of frequent recombination between chromosomal telomeres (157) and between plasmids and chromosomes (98) that generate new combinations of telomeric repeats. One possible intermediate in these rearrangements is an autonomously replicating circular Y' element that presumably arises from a recombination between tandemly repeated Y' elements (156). Since these circular molecules have no centromere, their copy number is expected to increase in the cells that harbor them, and the increased copy number could be stabilized by their integration back into telomeric regions.

Telomere Replication

Several different mechanisms that protect the ends of linear DNA molecules and allow their replication have evolved. These include protein primers, hairpin loops, untemplated addition of telomeric repeats by a terminal transferase, and, possibly, recombination. Mammalian adenoviruses, *Bacillus subtilis* phage ϕ29, and the *K. lactis* killer plasmids have a protein covalently linked to each of their 5' termini. These proteins serve as primers for DNA replication by covalently binding the initiating nucleotide (69, 200, 201, 293, 315, 326, 371, 394).

In vaccinia virus and *Paramecium* mitochondrial DNA, one or both ends are cross-linked by hairpin loops. DNA replication forks proceed around the loop, yielding a dimer molecule which can be cut to generate daughter monomers (18, 125, 311). If the site-specific nuclease that resolves the daughter molecules makes a staggered cut in the two strands (19) or recognizes the hairpin that can form by branch migration (369), then molecules with hairpin ends can be regenerated.

Neither of these mechanisms of telomere replication can easily account for all features of yeast telomeres, including their variable length, the de novo addition of yeast telomeres to plasmids carrying telomeres from other organisms, and the existence of genes that affect telomere length. Therefore, it is likely that yeast telomeres are replicated by a different mechanism.

The telomeres of *Tetrahymena* macronuclear DNA consist of a variable-length tract of a repeating hexanucleotide, $C_4A_2 \cdot T_2G_4$ (reviewed in reference 30). Individual telomeres contain between 20 and 70 repeats of this sequence and have one or more single-strand interruptions in each strand. The termini are protected from digestion by S1 nuclease, implying that they are either hairpins or protected by a tightly bound protein. A terminal transferase activity that adds telomeric repeats onto telomeric sequences has recently been identified in *Tetrahymena* sp. (131, 132). This enzyme is a ribonucleoprotein that specifically recognizes single-stranded oligonucleotides representing the G-rich strand of telomeric sequences from at least five different organisms and adds T_2G_4 sequences to them. The G-rich strands of several telomeric sequences can fold back on themselves and form a hairpin structure stabilized by guanine-guanine base pairs (145). Thus, the addition of single-stranded terminal repeats by the telomere terminal transferase generates a hairpin structure that can serve as a primer for synthesis of the C-rich strand. Telomere length is then determined by the equilibrium between shortening due to incomplete replication and lengthening by the telomere terminal transferase activity.

The similarity between yeast telomeres and *Tetrahymena* telomeres and the observations that the *Tetrahymena* telomere terminal transferase recognizes yeast telomeric se-

quences (131, 132) and that yeast cells add their own telomere repeats on plasmids carrying *Tetrahymena* telomeres (336, 391) are consistent with the idea that *S. cerevisiae* uses a similar mechanism for telomere replication. However, the evidence is all indirect at this point, and a yeast telomeric terminal transferase with the expected properties has not yet been identified.

Finally, two similar models which invoke a recombinational mechanism for the replication of telomeres and can also account for the known properties of yeast telomeres have been postulated (387, 391). The essential feature of these models is the repair or extension of the single-stranded 3′ end following its invasion into a homologous duplex region (reviewed in reference 390). There is no direct evidence that this mechanism is used for telomere replication in any organism, but it is clear that bacteriophage T4 uses a similar mechanism for the initiation of DNA replication (262).

Whether yeast telomeres are replicated by untemplated addition of terminal repeats or by a mechanism requiring recombination remains to be determined. In addition, several other issues remain. For example, how are broken chromosomes healed? One possibility is that broken ends are degraded to expose short sequences that resemble telomeric sequences. Walmsley et al. demonstrated that internal sequences as well as telomeres hybridize to a polyguanylate-polythymidylate probe, and these internal sequences could be suitable substrates for telomere addition (393). Another unresolved issue is how the equilibrium between shortening and lengthening is set. Clearly, multiple gene products could play a role, but how they interact is unknown.

MITOTIC CHROMOSOME SEGREGATION

Artificial Chromosomes

Natural yeast chromosomes are replicated and segregated efficiently during vegetative growth. Individual chromosomes are lost at a rate of approximately 10^{-5} per cell division (105, 142, 256, 363). In contrast, circular and linear artificial chromosomes constructed from bacteriophage or *E. coli* plasmid DNA and containing one or more *ARS* elements, a centromere, one or more selectable genes, and, in the case of linear molecules, telomeres are much less stable.

For both circular and linear constructs, stability increases with size (Fig. 6). Circular plasmids of <10 kb in size are lost at rates of 2×10^{-2} to 5×10^{-2} per division, and the loss rate decreases about 10-fold for a plasmid of about 100 kb (79, 114, 149, 266). Further increases in circular plasmid size result in small increases in loss rate, perhaps because sister chromatid exchange yields dicentric plasmids (149; see next subsection). Even the smallest circular constructs tested are maintained at one to two copies per cell, and for these plasmids the ratio of 1:0 segregations (simple losses)/2:0 segregations (nondisjunctions) is about 5:1 (149, 212).

The addition of telomeres to small centromere-containing plasmids drastically alters their behavior. In contrast to small circular plasmids, a 9-kb linear plasmid is not maintained stably (416). Linear plasmids of approximately 15 kb are lost at a rate of approximately 10^{-1} per cell division (87, 149, 265–267) a 10-fold higher rate of loss than observed for circular plasmids of the same size. Surprisingly, these linear constructs are maintained at 10 to 50 copies per cell (87, 266). The stability of linear constructs increases with increasing length (Fig. 6). The most stable artificial linear chromosome that has been examined is lost at a rate of approximately 10^{-3} per division, 2 orders of magnitude higher than natural chromosomes. The copy number of linear artificial chromosomes also decreases with increasing length, with 55-kb constructs being maintained at one to three copies per cell.

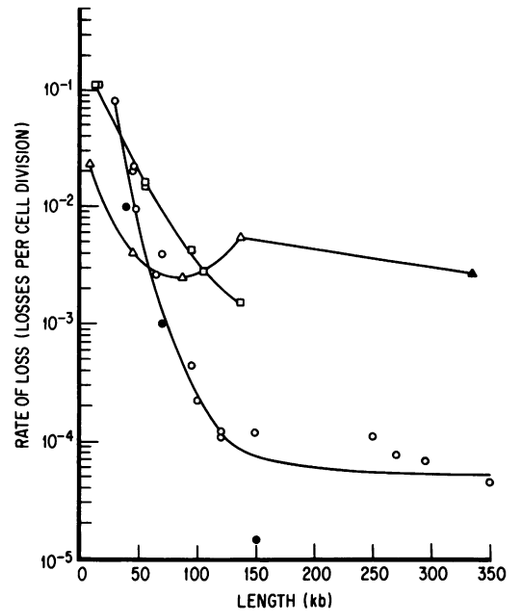

FIG. 6. Stability of linear and circular chromosomes. Curves relating chromosome stability to length are plotted for circular minichromosomes (▲, △), artificial linear chromosomes (□), and derivatives of chromosome III (●, ○). The sizes of chromosome III derivatives given in reference 265 have been corrected to conform with more recent estimates of the size of chromosome III. Symbols: △, circular minichromosomes; ▲, 335-kb circular derivative of chromosome III (118); □, artificial linear chromosomes (149, 266); ○, chromosome III (363); ●, chromosome III (265).

The strikingly high copy number and instability of short linear artificial chromosomes are properties that suggest that the inheritance of such chromosomes is via random segregation rather than the ordered segregation observed for circular centromeric plasmids and natural chromosomes. The segregation defect is not the result of permanent centromere inactivation. When linear plasmids were recovered from *S. cerevisiae* and circularized, the circular plasmids showed the expected stability and segregation properties (87, 266). In addition, linear dicentric plasmids were structurally unstable and gave rise to rearrangements that deleted one of the centromeres, as observed for dicentric circular plasmids (265). This result demonstrates that the centromere is functional in the linear plasmid. Thus, some characteristic of short linear chromosomes other than failure of centromere function must account for their random segregation.

Natural Chromosomes

Two differences between linear artificial chromosomes and natural chromosomes that could account for the instability of artificial constructs are length and position of the centromere. The shortest yeast chromosome, chromosome I, is approximately 250 kb in length, while the longest artificial construct tested is about half that size. In addition, genetic mapping indicates that natural yeast chromosomes are metacentric (261), while the artificial constructs by

necessity have their centromeres closely associated with one or both telomeres. By altering natural chromosomes, it has been possible to test the effects of both length and centromere position on the stability of chromosomes.

Telocentric derivatives of natural chromosomes, constructed by deleting an arm, splitting a chromosome near the centromere by recombination with a short artificial chromosome, or integrating an inverted telomere repeat near the centromere of a circular chromosome, are only three- to fivefold less stable than the wild-type chromosomes from which they were derived (269, 363, 364, 416). These results demonstrate that the instability of the artificial constructs cannot result from the close spacing of their centromeres and telomeres.

However, length appears to be an important determinant of chromosome stability. Deletion derivatives of chromosome III shorter than approximately 100 kb have greatly increased loss rates, with loss rate increasing exponentially with decreasing size (Fig. 6) (265, 363). Derivatives larger than about 100 kb have mitotic stabilities similar to that of the full-length chromosome. In addition, in a strain carrying a mutation that causes an increase in the rate of chromosome loss, *chl1*, the frequency of loss of individual chromosomes increases with decreasing chromosome size (229). Similarly, when nuclear fusion is prevented in zygotes by the *kar1* mutation, chromosomes are occasionally transferred between nuclei in the heterokaryon. In this situation the frequency of transfer of chromosomes varies inversely with their size (99).

Although length is an important determinant of natural chromosome stability, artificial linear chromosomes are still at least an order of magnitude less stable than natural chromosomes of equivalent length (Fig. 6). For short derivatives of natural chromosomes, most of the loss events are the result of nondisjunction (2:0 segregation). Interestingly, the decrease in stability of artificial constructs compared with natural chromosomes is the result of an increase in 1:0 segregations (265). One obvious difference between the artificial constructs and natural chromosome derivatives is the number of *ARS* elements. The artificial constructs examined carry three *ARS* elements, a yeast chromosomal *ARS* (usually *ARS1*), and *Tetrahymena ARS* elements associated with the telomeres. The shortest derivatives of natural chromosomes carry three or four *ARS* elements, but as their size increases, the number of *ARS* elements increases as well. However, the present data are not adequate to establish whether the decreased stability of artificial constructs is the result of their failure to replicate or simply of their loss from the nucleus.

While chromosome length is a major factor in chromosome stability, proper packaging of chromosomal DNA also appears important for chromosome function. Meeks-Wagner and Hartwell have shown that, when the normal stoichiometry of histone proteins is perturbed by overproducing either H2A and H2B or H3 and H4, the rate of chromosome loss is increased by 1 to 2 orders of magnitude (256). On the basis of these observations, they were able to identify two additional DNA sequences, *MIF1* and *MIF2*, that act in *trans* to increase rates of chromosome loss when they are present on high-copy-number plasmids (257).

Models

Three different models for sister chromatid segregation have been proposed that account for the dependence of segregation fidelity on chromosome length as well as the relative instability of artificial constructs.

First, Murray and Szostak have proposed that the segregation of sister chromatids is directed by catenation of daughter DNA molecules produced during DNA replication (268). The mitotic spindle stably attaches only to those sister chromatids that are physically linked. When replication forks meet within topologically closed domains, the daughter molecules become catenated (24, 361). Therefore, chromosomal DNA molecules too short to include one or more topological domains and a number of replication origins are expected to be unstable. Artificial chromosomes of intermediate length could be at a disadvantage compared with natural chromosomes because they have fewer replication origins or because they lack the DNA sequences or the proper chromatin structure to set up stable topological domains.

A prediction of this model is that small circular minichromosomes should be catenated if they are isolated from cells arrested after S phase but before mitosis. Koshland and Hartwell analyzed the structure of a circular minichromosome in cells arrested in G2 phase by cell cycle mutations or a reversible microtubule inhibitor (211). Using isolation conditions that appeared to rapidly inactivate topoisomerase II, they found that the majority of the circular minichromosomes were decatenated at all of the G2 blocks, but that they were nevertheless able to segregate properly when the block was reversed. These results strongly suggest that catenation of sister chromatids is not required for proper mitotic segregation.

Second, it is possible that telomeres have to be separated by a minimum distance. There is substantial cytological evidence that telomeres associate with each other and with the nuclear membrane (reviewed in reference 2). If these associations are important for chromosome segregation, then short chromosomes would have difficulty segregating properly. In this model, the greater instability of long artificial constructs would have to be explained differently. For example, they might lack DNA sequences necessary for efficient retention in the nucleus or for efficient interaction with the replication apparatus.

Third, there may be *cis*-acting sequences other than *ARS* elements, centromeres, and telomeres that are important for chromosome stability. If such elements exist, then each chromosome must have several such elements. The deletion derivatives of chromosome III (265, 363) and the telocentric derivatives of chromosome IV (416) demonstrate that each chromosome arm must contain an adequate number of such elements for normal stability. It is possible that the segregation elements adjacent to the *HMR E ARS* (206) and *CEN4* (243) represent this class of elements.

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